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Japan Report

SCIENCE AND TECHNOLOGY

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JAPAN REPORT

SCIENCE AND TECHNOLOGY

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BIOTECHNOLOGY

BIO-PROBE DETECTION OF SPECIFIC DNA VIEWED

Tokyo BIO INDUSTRY in Japanese Nov 85 pp 64-71

[Article by Toyozoh Takahashi, Associate Professor, and Kenji Okuda, Professor, Department of Bacteriology, School of Medicine, Yokohama City University; First of two-part article on "Genetic Diagnosis"]

[Text] The identification of pathogens in infectious diseases as well as the diagnosis of cancer, genetic diseases, etc. are now about to be performed at the level of DNA. In this environment, we shall introduce the "Bio-probe technique" which does not use isotopes, in two parts, titled "Genetic Diagnosis for You." Part one will pertain to an explanation and the principle of reagents and equipment necessary for this technique as well as the preparation of biotin-labeled DNA probes and their purification.

1. Introduction

There are various techniques to label DNA, and all are important in the field of molecular biology. For example, when DNA synthesis precursors labeled with radioactivity are added to a suitable cell culture system and a virus is cultured in the presence of ^{32}P-inorganic phosphoric acid, the virus can be extracted as a ^{32}P-labeled virus from the infected cells. In addition, when a method called nick translation is used, labeled DNA with high radioactivity can be prepared in vitro using the exonuclease activity and polymerization activity of E. coli DNA polymerase I.

In recent years, in the field of genetic engineering where remarkable developments have been achieved, DNA labeled with a radioisotope is prepared using nick translation and used as probes in experiments such as southern hybridization, colony hybridization, plaque hybridization, etc.

As mentioned previously, nick translation uses the DNA synthesis mechanism of E. coli's DNA polymerase I, and instead of radioactively labeled nucleotides, it is also possible to use biotin-labeled nucleotides in the reaction system.

The bio-probe (biotin-labeled probe) technique introduced here skillfully uses the nick translation reaction, which is frequently used as a genetic manipulation technique as stated above. It first incorporates a biotin-labeled nucleotide into the DNA, hybridizes the probe with the target DNA, and develops a color with streptavidin, alkaline phosphatase, and NBT (Fig. 1).

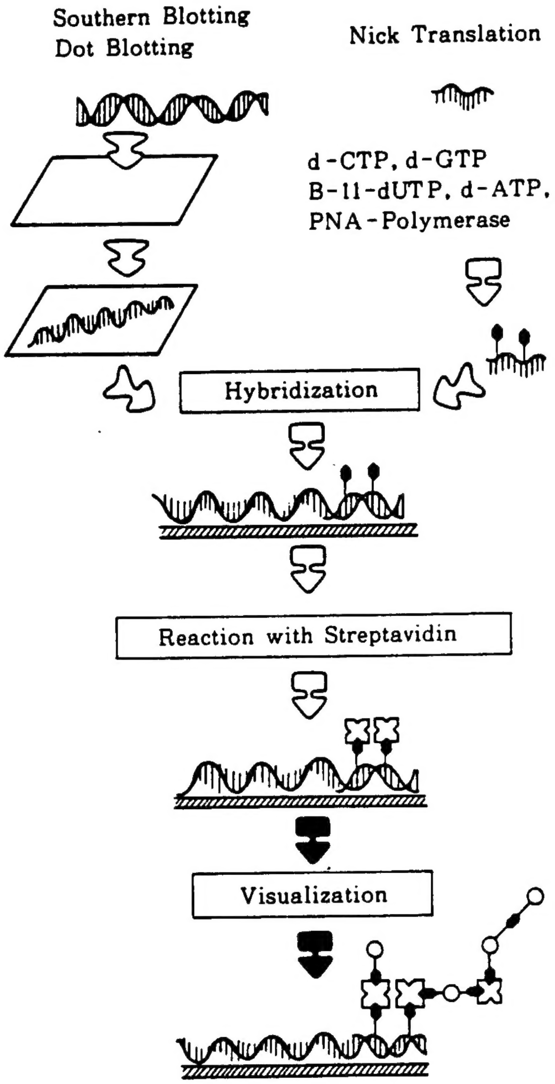

Southern Blotting
Dot Blotting
Nick Translation
d-CTP, d-GTP
B-11-dUTP, d-ATP,
PNA-Polymerase
Hybridization
Reaction with Streptavidin
Visualization

Fig. 1 Bio-probe technique

A special feature of this technique is that it eliminates a number of the problems involved in handling radioactively labeled substances by using a biotin-labeled nucleotide (biotin-11-dUTP). In addition, by using streptavidin and alkaline phosphatase instead of the conventional biotin-avidin-peroxidase detection method, it reduces the background contamination caused by non-specific binding and makes it possible to detect a nucleic acid sequence present even with a single labeled copy. Consequently, it is very suitable for in situ hybridization and is believed to be a technique highly applicable to the diagnosis of cancer, infectious diseases, and genetic diseases.

2. Materials and Methods

2.1 Reagents for DNA visualization

(1) Streptavidin storage solution
50 mM Tris-HCl (pH 7.5) (Sigma; Cat# T-1503)
0.2 mg/ml Sodium Azide (Sigma; Cat# S-2002)

Add streptavidin (Enzo Biochemicals or Bethesda Research Laboratories) to the above buffer in a ratio of 1 mg/ml.

(2) Biotin-bound polyalkaline phosphatase (BBpolyAP) storage solution
3 M NaCl
1 mM $MgCl_2$ (Merck; Cat# 5833)
0.1mM $ZnCl_2$ (Wako Pure Chemical; Cat# 265-00275)
30 mM Triethanolamine (pH 7.5)
(Wako Pure Chemical; Cat# 207-06155)

Add calf intestinal alkaline phosphatase (Bethesda Research Laboratories) bound to biotin to the above solution at a ratio of 1 mg/ml. If you need only calf intestinal alkaline phosphatase, it can be purchased from Boehringer Mannheim (Cat# 567-752).

(3) Nitro-blue tetrazolium (NBT) solution

Add NBT (Sigma; Grade III, Cat N-6876) at a ratio of 70% dimethyl formamide (Wako Pure Chemical; Cat# 043-02912).

(4) 5-Bromo-4-chloro-3-indolyl phosphate (BCIP)*

The substance is BCIP (Sigma) dissolved at a ratio of 50 mg/ml in dimethylformamide.

---

*5-bromo-4-chloro-3-indolyl phosphate (BCIP) p-toluidine salt (Sigma; Cat# B-8503)

BRL (Bethesda Research Laboratories, USA) sells the above-mentioned reagents, (1), (2), (3), and (4) in kit form in units of 120 µl, 60 µl, 660 µl, and 500 µl respectively. When these reagents are used, 20, 100 cm^2 filters can be stained.

(5) Stop TE buffer
20 mM Tris-HCl (pH 7.5)
5 mM EDTA

2.2 Reagents for DNA test strip preparation

(1) Biotin-bound DNA
200 pg/µl biotin-bound lambda DNA*
0.2 µg/µl sheared herring sperm DNA**
6 x SSC
*Lambda DNA is Wako Pure Chemical's Code# 314-00411,
**For the herring sperm DNA, Sigma, type VII is sheared.

(2) Buffer for DNA dilution
0.2 ug/µl sheared herring sperm DNA (Sigma, type VII)
6 x SSC

Biotin-bound polyalkaline phosphatase can be prepared by polymerizing through cross-linking of commercial calf intestinal alkaline phosphatase (Boehringer Mannheim; Cat# 567-752) to disuccinimidyl suberate and by binding biotin based on the method of Leary, et al.[2]. However, since Bethesda Research Laboratories sells one already bound which has stable quality, we recommend buying it. In Japan, Maruzen Oil is handling it.

The above-mentioned reagents are also sold by BRL as a DNA Detection System. If you can afford it, it is convenient to buy because it is systematized.

2.3 Reagents and apparatus for nick translation

(1) Biotin-11-dUTP solution (BRL; Cat# 9507SA)
100 mM Tris-HCl (pH 7.5)
0.4 mM Biotin-11-dUTP

(2) Solution A4
0.2 mM each of dATP, dCTP, dGTP (Sigma; Cat# D-6500, D-4760, D-4135)
500 mM Tris-HCl (pH 7.8) (Sigma; Cat# T-1503)
50 mM $MgCl_2$ (Merck; Cat# 5833)
100 mM 2-Mercaptoethanol (Sigma; Cat# M6250)
100 µg/ml nuclease-free BSA (Sigma, fraction V: Cat# A7030)

(3) Solution B
5 µg lambda phage DNA (Wako Pure Chemical: Code# 314-00411)
0.1 mM EDTA (Sigma: Cat# ED-255)
10 mM Tris-HCl Tris-HCl (pH 7.5) (Sigma: Cat# T-1503)
120 mM NaCl (Wako Pure Chemical: Cat# 191-011665)

(4) Solution C
0.4 units/µl DNA polymerase I (Bethesda Research Laboratories)
40 pg/µl DNAse I (Bethesda Research Laboratories)
50 mM Tris-HCl (pH7.5) (Sigma: Cat# T-1503)
5 mM Mg-Acetate (Merck: Cat# 5819)
1 mM 2-Mercaptoethanol (Sigma: Cat# 6250)
0.1 mM Phenylmethylsulfonylfluoride (PMSF) (Sigma: Cat# 7626)
50 % Glycerol (Merck: Cat# 4094)
100 µg/ml nuclease-free BSA (Sigma, fraction V: Cat# A7030)

(5) Solution D (Stop buffer)
300 mM Na_2 EDTA (pH 8.0) (Sigma: Cat# ED-255)

(6) Solution E
Sterile distilled water

Of the above, items (2)-(6) are sold by BRL as a Nick Translation Reagent Kit (Cat# 8160SB).

(7) Nitrocellulose filter (S&S Company)
(8) Vacuum oven (Ikemoto Rikagaku Kogyo)
(9) Sephadex G-50 (Pharmacia Fine Chemicals)

2.4 Buffers, etc.

(1) Buffer 1
0.1 M Tris-HCl (pH 7.5) (Sigma: Cat# T-1503)
0.1 M NaCl (Wako Pure Chemical: Cat# 191-01665)
2 mM $MgCl_2$ (Merck: Cat# 5833)
0.05 % (v/v) Triton X-100 (Wako Pure Chemical: Cat# 203-03215)

(2) Buffer 2 (3 g BSA/100 ml Buffer 1)
3 g Bovine serum albumin (Sigma, fraction V: Cat# A7030)
up to 100 ml with Buffer 1

(3) Buffer 3
0.1 M Tris-HCl (pH 9.5) (Sigma, Cat# T-1503)
0.1 M NaCl (Wako Pure Chemical: Cat# 191-011665)
50 mM $MgCl_2$ (Merck: Cat# 5833)

(4) 20 x SSC
3.0 M NaCl (Wako Pure Chemical: Cat# 191-011665)

0.3 M Sodium citrate (pH 7.0) (Merck: Cat# 6448)

(5) 5 % SDS
Sodium dodecylsulfate 5g/100ml (Bio Rad: Cat# 161-0301)

(6) 1 x SSC + 0.1 % (w/v) SDS (buffer for the column)
0.15 M NaCl (Wako Pure Chemical: Cat# 191-011665)
0.015 M Sodium citrate (pH 7.0)
0.1 % (w/v) SDS (Bio Rad: Cat# 161-0301)

(7) 5-10 ml plastic pipettes
(8) silicon-treated glass wool
(9) pinch cock
(10) Silicon rubber tubes (ϕm)
(11) 1.5 ml Eppendorf tubes
(12) scissors
(13) rulers, pencils
(14) micropipetter and tips
(15) filter papers (Whatman 3 MM)

3. Procedures

Briefly stated, the procedures consist of the following four steps.

1) Biotin-11-dUTP-labeled DNA probe is prepared by means of the nick translation process.
2) The above-mentioned biotin-labeled probe is hybridized with the target DNA immobilized on a nitrocellulose membrane.
3) Streptavidin and a biotin-labeled alkaline phosphatase polymer are bound to 2) by incubation.
4) By adding the substrates (color developer), i.e., NBT and BCIP to 3), alkaline phosphatase reacts detecting the target DNA sequence on the nitrocellulose membrane as blue dots or bands.

We shall now describe the procedure step-by-step in detail.

3.1 Preparation of biotin-labeled DNA probe

3.1.1 Nick translation

Biotin-11-dUTP is a deoxynucleotide triphosphate similar to dTTP, and it is efficiently incorporated into DNA by the nick translation reaction of DNA polymerase I in the presence of dATP, dGTP, and dCTP. As shown in Fig. 2, in the case of biotin-11-dUTP, since the biotin is bound to the base, uracil, of 2'-deoxyuridine 5'-triphosphate via allylamine and aminocaproic acid, when it is substitituted with thymidine triphosphate (TTP) and incorporated into the DNA, the DNA constructed is labeled with biotin as a result.

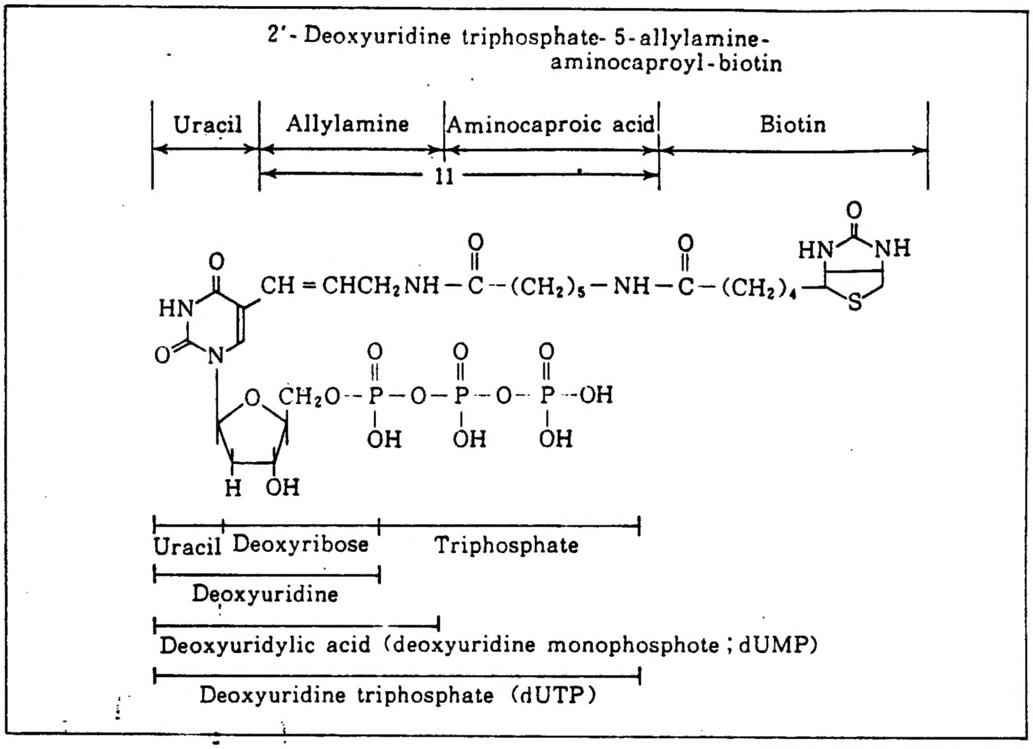

2'- Deoxyuridine triphosphate- 5-allylamine-aminocaproyl-biotin
Uracil
Allylamine
Aminocaproic acid
Biotin
11
Uracil
Deoxyribose
Triphosphate
Deoxyuridine
Deoxyuridylic acid (deoxyuridine monophosphote ; dUMP)
Deoxyuridine triphosphate (dUTP)

Fig. 2 Biotin-11-dUTP structure

Consequently, the detection sensitivity of the biotin-labeled DNA probe is greatly influenced by the degree of nucleotide substitution in this labeling reaction process.

Fig. 3 shows a flow diagram of the procedures.

*Caution

1) In the cases of homopolymer tailing using terminal deoxynucleotidyl transferase or substitutional synthesis using T_4 DNA polymerase, the same method can be used.

2) In order to carry out the nick translation reaction with high yield, the desirable concentrations of dATP, dCTP, dGTP, and biotin-11-dUTP are about 20 μM in each case.

3) The rate of biotin-11-dUTP incorporation can be determined by purifying the labeled DNA probe and visualizing it in parallel with the later-mentioned test strips for detection. Or, by having 5 μCi of freeze-dried [^{3}H]-dATP in an Eppendorf tube before adding the nick translation reagent, the degree of biotin-11-dUTP incorporation can be estimated from the rate of ^{3}H-labeled substance being incorporated.

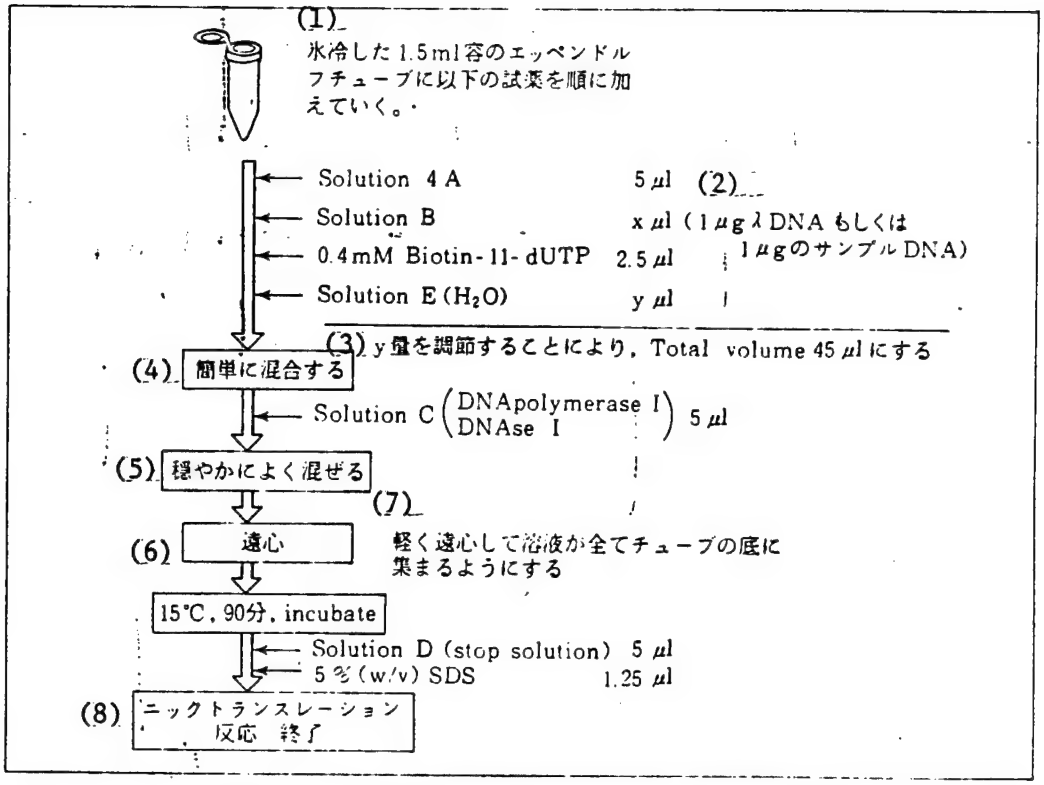
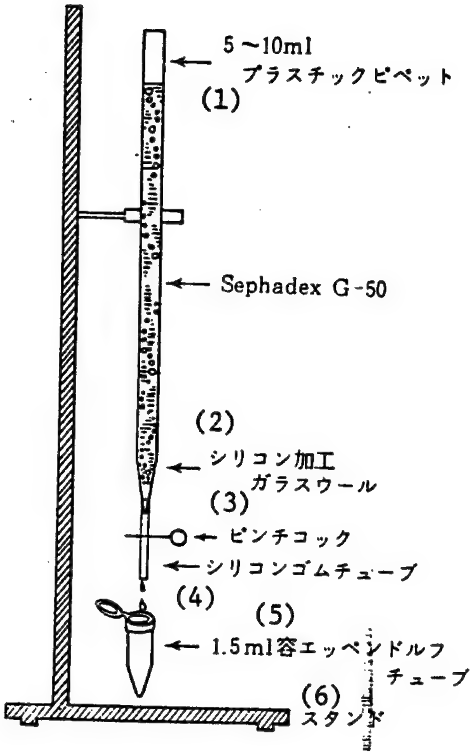

(1)
氷冷した1.5ml容のエッペンドルフチューブに以下の試薬を順に加えていく。
Solution 4 A
5 µl
(2)
Solution B
x µl（1µg λ DNA もしくは 1µgのサンプルDNA）
0.4mM Biotin-11-dUTP
2.5 µl
Solution E (H₂O)
y µl
(3) y量を調節することにより，Total volume 45 µl にする
(4) 簡単に混合する
Solution C (DNApolymerase I / DNAse I) 5 µl
(5) 穏やかによく混ぜる
(7)
(6) 遠心
軽く遠心して溶液が全てチューブの底に集まるようにする
15℃，90分，incubate
Solution D (stop solution) 5 µl
5％(w/v) SDS 1.25 µl
(8) ニックトランスレーション反応 終了

Fig. 3 Nick translation reaction

key: 1) Add the following reagents in order into the chilled 1.5 ml Eppendorf tube.
2) (1 µg lambda DNA or 1 µg sample DNA)
3) By regulating the y volume, make the total volume 45 µl.
4) Briefly mix
5) Gently mix well
6) Centrifugation
7) Centrifuge lightly so that the entire solution collects at the bottom of the tube.
8) Completion of nick translation reaction

3.1.2 Purification of biotin-labeled DNA probe

It is necessary to separate the biotin-labeled DNA probes from the free biotin-11-dUTP which was not incorporated. In the past, we separated and purified ^{32}P-labeled DNA probes with column chromatography using Sephadex G-50 - 75. It is possible to separate and purify biotin-labeled DNA probes from free biotin-11-dUTP by using the same method. Although separation and purification are possible by ethanol precipitation, we shall discuss exclusion chromatography here. This is a method to recover the labeled DNA probes rapidly and quantitatively by gel filtration with Sephadex G-50. First, as shown in Fig. 4, a Sephadex G-50 column is prepared by packing a 5 - 10 ml plastic pipette with silicon-treated glass wool. Have the Sephadex G-50 saturated with 1 x SSC + 0.1 % (w/v) SDS solution shown in section 2.4 (6) under materials and methods.



key: 1) plastic pipette
2) silicon-treated glass wool
3) pinch cock
4) silicon rubber tube
5) 1.5 ml Eppendorf tube
6) stand

Fig. 4 Purification of biotin-labeled DNA

The use of NACS resin is not recommended.

1) When the column is ready, layer a sample with completed nick translation reaction on the column. Open the pinch cock, and collect 150 µl fractions into 1.5 ml capacity Eppendorf tubes.

By having SDS added to the elution buffer at a concentration of 0.1 %, it is possible to prevent non-specific binding of the biotin-labeled DNA probe to the plastic tube and increase the yield.

2) Spot 1-2 µl of each fraction onto a nitrocellulose filter and incubate for 30 minutes at 80°C in a vacuum oven.

3) Detect biotin-labeled DNA on the nitrocellulose filter according to the method described later in sections 3.1.3 and 3.1.4. In other words, the idea is to visualize the spotted filter in order to determine in which fraction the labeled DNA is located. When color develops too fast or the pigment is

too intense to distinguish the DNA peak, dilute 1 µl of each fraction with the column buffer to 20 µl and repeat from item 2).

4) When necessary, the yield of the biotin-labeled DNA probe can be quantitated by diluting some of the pooled fractions with DNA dilution buffer, spotting them on a nitrocellulose filter and visualizing along with the DNA test strips.

Although this technique is slightly more troublesome since monitoring with a Geiger counter is not possible as in the case of radioisotope-labeled probes, once separated and purified, it can be stored for several month at -20°C unlike the radioisotope-labeled probes.

*Caution

When biotin-labeled DNA probes are phenol-extracted, since there is a possibility of their migrating to the phenol layer, it is better not to extract with phenol.

3.1.3 Testing biotin-labeled DNA probe

When testing biotin-labeled DNA probes for research, it is necessary to have biotin-bound DNA control as a standard. For a standard, we labeled the DNA extracted from lambda phage with biotin using the nick translation reaction and prepared sheared herring sperm DNA as described in the section 1) as a reagent for DNA test strip preparation.

1) First, dilution was performed as follows using five 1.5 ml Eppendorf tubes (Fig. 5).

2) Next, have ready a 10 cm x 5 cm nitrocellulose filter, section it with a pencil into 1 cm squares and cut with scissors into 10 strips (1 cm x 5 cm). Spot each section on a strip with 5 µl of each diluted solution a - e of 1). The last solution e is only the buffer for DNA dilution and is used as a control (Fig. 6).

3) Dry the filter in a vacuum oven for 1-2 hours at 80°C. Dried strips can be stored in a desiccator for several months. By staining these strips one at a time along with the nitrocellulose filter used in the experiment, the sensitivity of DNA detection can be determined.

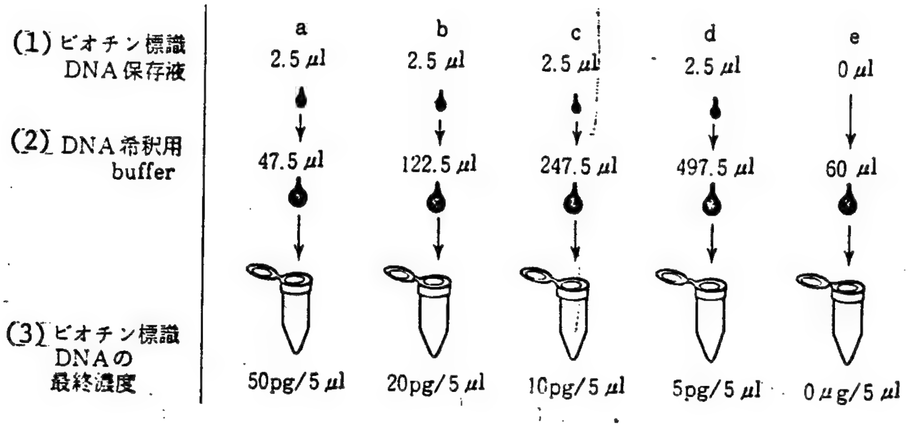

(1) ビオチン標識 DNA保存液
(2) DNA希釈用 buffer
(3) ビオチン標識 DNAの 最終濃度
a
b
c
d
e
2.5 µl
2.5 µl
2.5 µl
2.5 µl
0 µl
47.5 µl
122.5 µl
247.5 µl
497.5 µl
60 µl
50pg/5 µl
20pg/5 µl
10pg/5 µl
5pg/5 µl
0 µg/5 µl

Fig. 5 Dilution of biotin-labeled DNA

key 1) biotin-labeled DNA storage solution
2) DNA dilution buffer
3) final concentration of biotin-labeled DNA

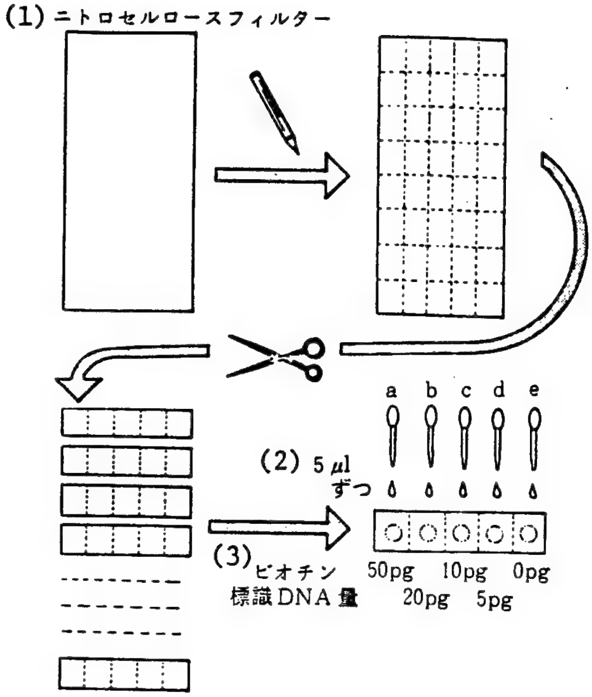

(1) ニトロセルロースフィルター
a
b
c
d
e
(2) 5 µl ずつ
(3) ビオチン標識DNA量
50pg
10pg
0pg
20pg
5pg

Fig. 6 Preparation of test strips

key 1) nitrocellulose filter
2) 5 µl each
3) biotin-labeled DNA

References

1) Langer, P. R.; Waldrop, A. A and Ward, D. C.: *Proc. Natl. Acad. Sci. USA*, 78, 6633–6637 (1981)

2) Leary, J. J., Brigati, D. J. and Ward, D. C.: *Proc. Natl. Acad. Sci. USA*, 80, 4045-4049 (1983)

3) Wahl, G. M., Stern, M. and Stark, G. R.: *Proc. Natl. Acad. Sci. USA*, 76, 3683–3687 (1979)

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CSO: 4306/1091

BIOTECHNOLOGY

INDUSTRIAL TECHNOLOGIES FOR ASPARTAME PRODUCTION VIEWED

Tokyo BIOINDUSTRY in Japanese Sep 85 pp 5-11

[Article by Kiyotaka Oyama, chief, Development Production Department, Toyo Soda Industries, (KK): "Trends in the Technology Development of Aspartame Manufacturing"]

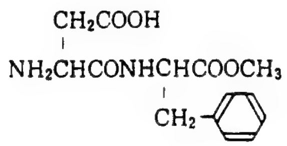
[Text] The chemical synthesis of and various other Aspartame manufacturing technologies and their related problems have been studied on the basis of the patent and other documents.

1. In the Beginning

Aspartame (APM) is a peptide type sweetener which is composed of L-Asparagine acid (L-Asp) and L-phenylalanine (L-Phe). In 1965, during synthesis research on Gastrolin which is a bone liquid secretion hormone, at the G.D. Searle Company in the United States, Aspartame was accidentally discovered to have strong sweetening capabilities.(1) It has about 200 times the sweetening power of sugar and has sweetening qualities similar to sugar. There is no disagreeable aftertaste. Compared to sugar, it is relatively difficult for it to cause tooth decay and while having the same sweetening capacity as sugar it has only 1/200th of the calories so its demand, particularly in the United States, has risen dramatically as a diet sweetener.

Because it is structured from L-Asp which is an essential amino acid and from L-Phe, it is believed to have little toxicity but the U.S. FDA [Food and Drug Administration] conducted long-term safety tests from such a variety of aspects that it created the statement "There has never been any other product that has undergone such extensive safety tests in FDA history." Today, in almost all of the advanced nations, some 30 or more, including the United States, Canada, Europe, Australia and Japan its use is authorized.

The APM structure is as shown below and G.D. Searle obtained patents in some 39 major countries of the world for its use as a sweetening agent. However, the protection of the patents will expire around 1987 in almost all of these countries except the United States (in the United States the patent rights have been extended to 1992).


CH₂COOH
NH₂CHCONHCHCOOCH₃
CH₂

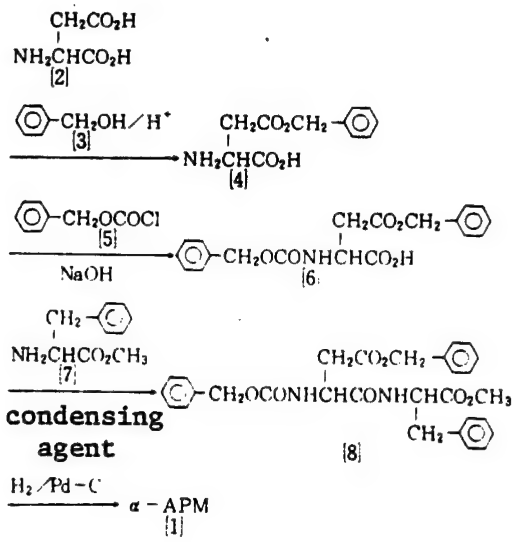
[1]

In the manufacturing of APM there are two methods, the chemical synthesis method and the bioscience method. Each of the various methods have a number of patent applications pending.

2. Chemical Manufacturing Method

The chemical manufacturing method in almost all cases involves the synthesis method utilizing peptide synthesis which has been used in the past and there is nothing particularly new in this process. As with the other processes the major points in the technology development in this sector insofar as the industrial process is concerned is with the question of how much the creation of by-products can be limited and to what extent the objective product can be made efficiently as well as how much of the manufacturing process can be abbreviated in order to reduce facilities expenses. Also, of importance is the question of how effectively the by-products can be removed in order that a high purity APM can be obtained. In the chemical type manufacturing method, since it can only use the expensive L body amino acid, basic raw materials are retrieved from both the raw materials which had not been spent as well as from the by-products created in order to reduce the cost of the raw materials. Furthermore, during the reaction process or in the retrieval process it is necessary that due precaution be exercised in order that there is no racemization of the amino acid.' There are many processes reported patent-wise and some of the more representative ones are shown below.

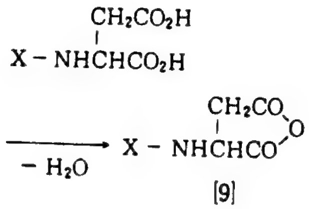
2.1 [2)–4)]


CH₂CO₂H
NH₂CHCO₂H
[2]
CH₂OH / H⁺
[3]
CH₂CO₂CH₂
NH₂CHCO₂H
[4]
CH₂OCOCl
[5]
NaOH
CH₂CO₂CH₂
CH₂OCONHCHCO₂H
[6]
CH₂
NH₂CHCO₂CH₃
[7]
condensing agent
CH₂CO₂CH₂
CH₂OCONHCHCONHCHCO₂CH₃
CH₂
[8]
H₂ / Pd-C
α - APM
[1]

This method is the most orthodox of the peptide synthesis methods. First of all Asp [2] β-carboxyl base is allowed to react with benzyl alcohol [3] existing under acid and this is protected as β-benzyl ester. Next, [4] is allowed to react with carbobenzoxyl chloride [5] and the base is protected and after it is made into [6], under the reduced N, N-zinchlorhexylcarbodimyd, it is allowed to react with phenylalaninemethylester [7] and [8] is achieved. Then, under a restorative catalytic agent such as Pd-C it is allowed to react with hydrogen to remove the benzyl and carbobenzoxyl bases (Z-base) to obtain APM [1].

According to this method, it is definitely certain that only α-APM will be produced. However, the esterization of the β-carboxyl base and because of the need to use expensive condensing agents, in terms of an industrial production method which places importance on economy, it is not a method in which satisfaction is inherent.

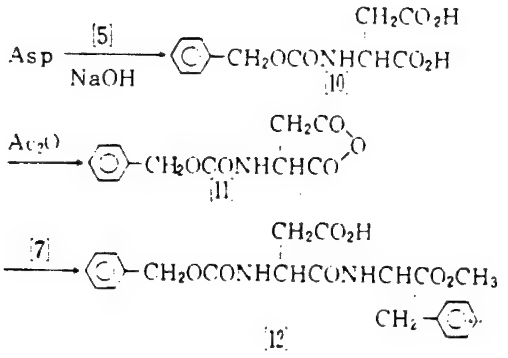
Therefore, there are many methods being developed to overcome these shortcomings. The common denominator in these methods is that 2 of the carboxyl bases of the Asp are anhydrated and ringed and an anhydrous molecular acid product [9] is produced (Z is the protective base for the amino base).

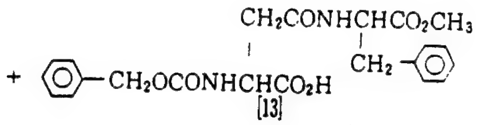


[9]

With [9] reacting with [7] the ring is opened and G peptide is given off. According to this sort of method the shortcomings mentioned earlier can be overcome. However, since there is no great difference between the 2 carboxyls, it is not possible to avoid the creation of the by-product β-APM which has some bitterness. Therefore, the separation of these metamers becomes necessary. Also, because β-APM is produced in large volumes the retrieval of the amino acid raw materials becomes very important from an industrial point of view.

2.2 5)-7)

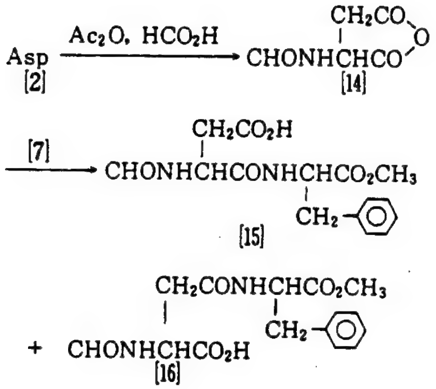




[13]

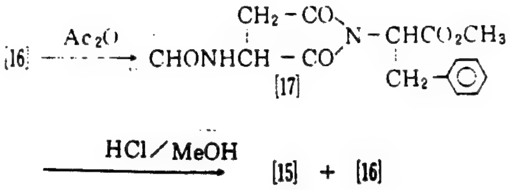
In this method the amino base of the Asp is protected with Z base and after it is made into N-benzyloxyCarboniasparagine acid [10], it is ringed with a dessicant such as acetic anhydride and a dehydrated acid [11] is obtained. Further, by allowing it to react with [7] a compound of the α body [12] and β body [13] of APM which has been protected by the Z base is obtained.

2.3 8)-13)

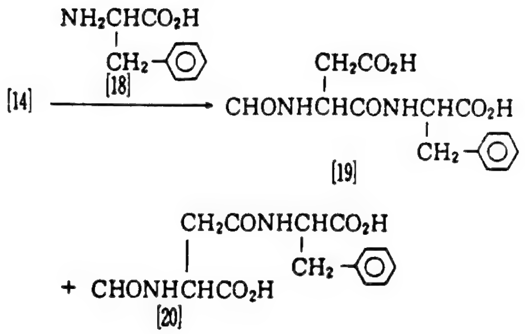


In 2.2 the Z step in the process and the cyclode hydration process were each separate but if Asp is allowed to react with formic acid and acetic anhydride the introduction of the formyl base (CHO base) into the amino base and the cyclode hydration will occur at the same time. Therefore, it is advantageous in the reaction process and, furthermore, because safe reaction testing materials can be used, it is the more appealing method. When [14] and [7] are permitted to react, the compound α body [15] and β body [16] of CHO-APM is given. At this point, after the β body is separated, either the CHO base is separated or it is separated as a compound and the β body is then separated and α-APM is obtained.

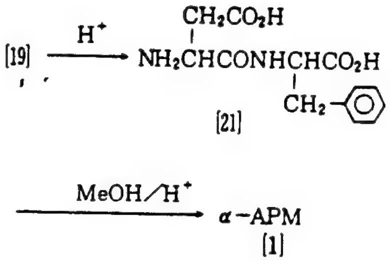
The β body CHO-APM [16] which has thus been separated is allowed to react with acetic anhydride as shown below and, as asparatimid [17] it is further treated with methanol to open the ring with acid and a conversion to a compound of α body and β body is formed.



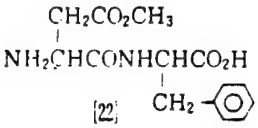
The big problem in this method is that in the separation of the CHO base a portion of the ester chain is caused to break.


NH₂CHCO₂H
CH₂
[18]
[14]
CH₂CO₂H
CHONHCHCONHCHCO₂H
CH₂
[19]
CH₂CONHCHCO₂H
CH₂
+ CHONHCHCO₂H
[20]

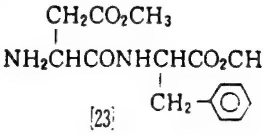
Therefore, as shown in the above, phenylalanine [18] is allowed to react with with an anhydrous molecular acid product [14] and a compound of α body [19] and β body [20] that does not have ester coupling is produced. Further, a new process has been developed in which after [19] and [20] have been separated the CHO base is separated as shown in the next step and the G peptide which has been obtained is methyl esterized and α-APM is obtained.


[19]
H⁺
CH₂CO₂H
NH₂CHCONHCHCO₂H
CH₂
[21]
MeOH／H⁺
α-APM
[1]

However, in this instance, control of the reaction is difficult and in addition to α-APM [1], esterized side chain carboxyl base products [22] and G methyl esters [23] are produced. And, some nonreacting elements [21] exist so these must be separated.


CH₂CO₂CH₃
NH₂CHCONHCHCO₂H
CH₂

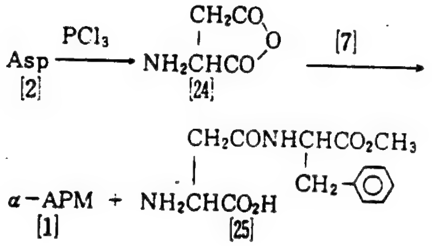
[22]


CH₂CO₂CH₃
NH₂CHCONHCHCO₂CH₃
CH₂

[23]

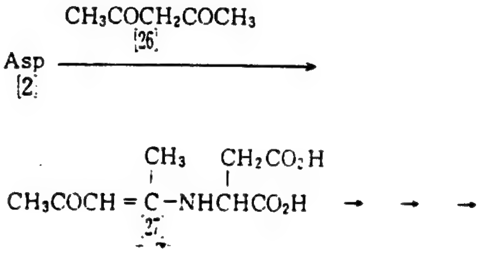
2.4(14-15)

In all of the above methods the amino base of the Asp was protected by some means or other. However, as shown below, a process has been developed where the anhydrous molecular acid product [24] is linked without protection and this is allowed to react with [7] and α-APM is obtained directly.


PCl₃
Asp
[2]
CH₂CO
O
NH₂CHCO
[24]
[7]
CH₂CONHCHCO₂CH₃
CH₂
α-APM + NH₂CHCO₂H
[1]
[25]

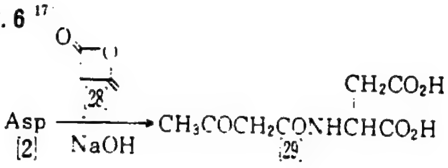
According to the method, while the various problems related to the production of a compound of α body [1] and β body [25] still remain, the abbreviation of the production steps through the introduction of the protection base and separation, makes the reaction process time very short and is very welcome as well from the standpoint of materials expenses. However, because of the fact that the Asp of the amino base is not protected there is strong reaction characteristic of [24]. As a result, during condensation, a variety of subreactions are likely to take place so it is necessary that the resulting by-products be effectively separated.

2.5 [16]


CH₃COCH₂COCH₃
[26]
Asp
[2]
CH₃ CH₂CO₂H
CH₃COCH = C-NHCHCO₂H
[27]

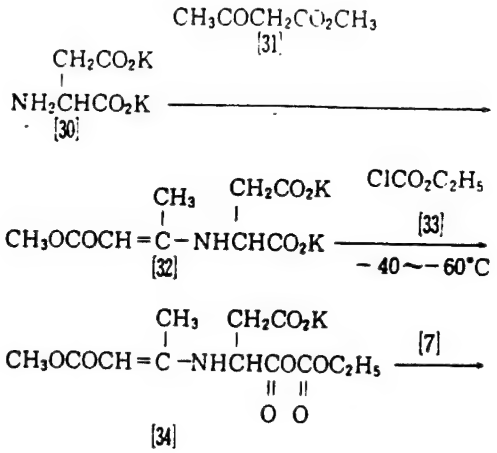
In this method the Asp's amino base is protected with 1-methyl-2-acetylvinyl and allowed to react with acetyl acetone [26] and after obtaining [27] it is anhydrated and then condensed with [7] which results in a G peptide compound of α body and β body. The separation protection reaction is accomplished with hydrochloric acid. The problem with this method is the use of a very expensive protective base.

2.6 [17]


[28]
Asp
[2]
NaOH
CH₂CO₂H
CH₃COCH₂CONHCHCO₂H
[29]

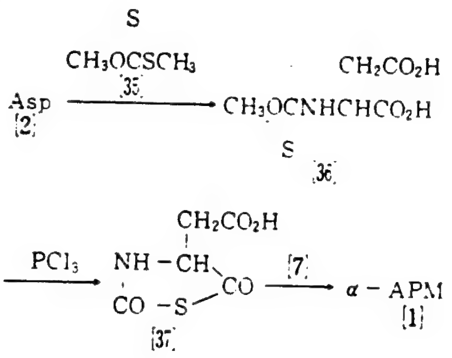
In this method Asp is allowed to react with G ketene [28] under the presence of caustic soda and the amino base is protected with acetyl acetone. After [29] is obtained the standard procedures are followed to anhydrate it and then this is condensed and this results in obtaining the α body and β body G peptide compound. The acetyl acetone base is separated using hydroxylamine.

2.7 [18]


CH3COCH2CO2CH3
[31]
CH2CO2K
NH2CHCO2K
[30]
CH3
CH2CO2K
ClCO2C2H5
[33]
CH3OCOCH=C-NHCHCO2K
[32]
- 40~- 60°C
CH3
CH2CO2K
[7]
CH3OCOCH=C-NHCHCOCOC2H5
O O
[34]

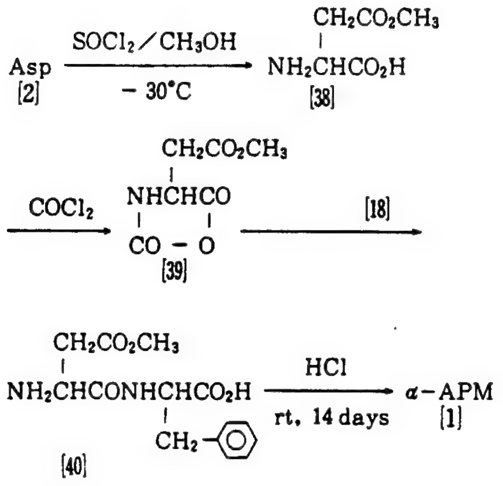
In this method, the potassium hypochlorite salt of the Asp [30] is allowed to react with aceto acetic acid ester [31] to protect the amino base. Then, it is allowed to react with chloroformicetyl [33] and a mixed acetic anhydride [34] is obtained. After this is allowed to react with [7] a protective base extraction reaction is undertaken with triethylamine at -30°C to -40°C and APM is obtained. The problem with this method is the need to use expensive testing chemicals as well as the severe reactional parameters.

2.8 [19]


S
CH3OCSCH3
[35]
Asp
[2]
CH2CO2H
CH3OCNHCHCO2H
S
[36]
CH2CO2H
PCl3
NH-CH
CO
CO-S
[37]
[7]
α-APM
[1]

In this method Asp is allowed to react with methylxanthogenic acid methyl [35] and the amino base is protected with N-methoxythiocarbonyl base. Then, this is ringed with phosphorous trichloride and after it is made into N-thiocarbonic anhydride, it is allowed to react with [7] and α-APM is obtained. According to this method α-APM can be obtained exclusively but there are such problems as the need to use expensive testing elements and the need to devise a means of removing undesirable odors from the product.

2.9 20)



Asp
[2]
SOCl2/CH3OH
- 30°C
CH2CO2CH3
NH2CHCO2H
[38]
COCl2
CH2CO2CH3
NHCHCO
CO - O
[39]
[18]
CH2CO2CH3
NH2CHCONHCHCO2H
CH2
[40]
HCl
rt. 14 days
α-APM
[1]

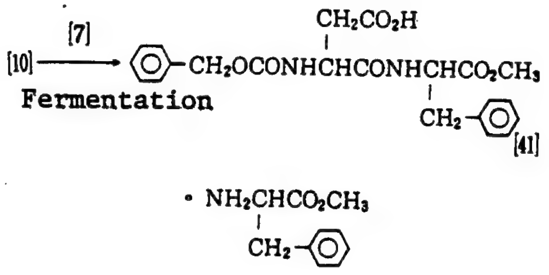
In this method Asp is allowed to react with thionylchloride under the presence of methanol and a β-methylester of Asp [38] is first obtained. This is made into N-carboxyacetic anhydride [39] by using phosgene and by allowing it to react with phenylalanine [18] and the β-methylester [40] of α-L-aspartyl-L-phenylalanine is obtained. Then, the β-methylester base of [40] is isomerized in hydrochloric acid methanol and α-APM is obtained. The problem area with respect to this method is that the isomer reaction requires too much time.

3. Bioscience Method

Many methods regarding fermentation or the production of microorganisms needed for fermentation, their cultivation medium and their disposal products have been reported.

3.1 (21-25)

Recently the peptide compounding using the reverse reaction of the hydrolytic dissociation reaction of peptide chains by protein degradation fermentation has been attracting attention. This method is a utilization of this process. In other words, the following sort of peptide chain reactions take place under protein degradation fermentation with its singular characteristics when such hydrophobic amino acid residual bases such as leucine and phenylalanine are used on the amino side base of the peptide chain.



[10]
[7]
Fermentation
CH2CO2H
CH2OCONHCHCONHCHCO2CH3
CH2
[41]
• NH2CHCO2CH3
CH2

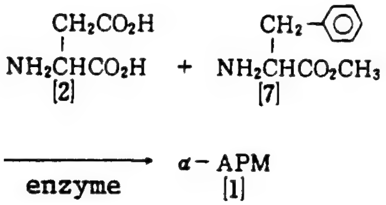
In this reaction, even if the racemized base characteristic is used, only L-L type G peptide of the L body which was the only thing that participated in the reaction, is given off and this selectively forms a supplement substance along with the [7] of the D body which has not yet reacted. Also, the β-carboxyl base of Asp which, despite being unprotected, only has a reaction with the α-carboxyl base base and no β body is produced. Further, there are superior characteristics such as being able to undertake reactions under mild conditions such as in an aqueous solution. A method which cannot be employed in the chemical compounding method.

$$[41] \xrightarrow{H^+} [12] + [7]$$

[41], as shown above is broken down into various components by acid. The [7] of D body which has been separated here is racemized and then reused as raw material. On the other hand, [12], through normal connected ringed reaction, causes the protected base to separate and α-APM is obtained. In this instance, there is no proliferation of the β-APM so the purification is extremely simple. Since APM's stability under heat is not very good during the purification process it is easily broken down into G ketopiparazene so, in comparison to the complicated purification process required in the chemical compounding method, this is more advantageous.

3.2(26-27)

In 2.1 since endopeptidaze is used as a protein degrading enzyme, in order to cause a condensing reaction, the amino base of the Asp needs to be protected with an appropriate protective base.(28) On the other hand, reaction will proceed without protection as in the following case using exopeptidizing which gives α-APM directly.

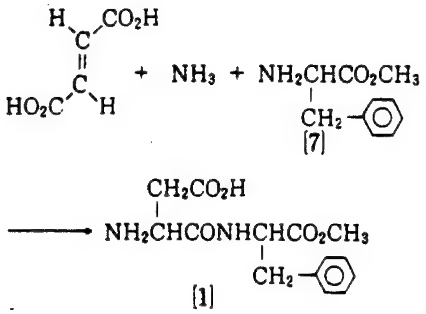


Reaction can be undertaken in the presence of such microorganisms as those which belong to pseudomonas, alcaligenes, torulopis, rhodotorula, sporobalomycetates,. achromobacter, corynebacterium, esherichieae, flavobacterium, saccharomyces, etc., or their culture products.

According to this method processes of the introduction of the protective base into the amino base of the Asp and the process of separation can be abbreviated and, because there is no β-APM regeneration, it is a very appealing method. However, the rate of return still appears to be low. The problem for the future is probably, how will the equilibrium be moved to favor the production side.

3.3(29)

A method has been reported whereby tumeric acid, ammonia and phenylalanine methylester are allowed to react with a microorganism of the pseudomonas variety or one of its cultivated products and this produces aspartame in one step (see next formula).



In this method, first of all, tumeric acid and ammonia react producing L-asparagene acid. Next, phenylalanine methylester reacts with this and α-APM is believed to be given off. α-APM can be compounded in one step with this method and it requires much less expensive raw materials than 2.2. However, at the present time, the rate of return of the product appears to be low.

4. Conclusion

In the foregoing, I have discussed the characteristics of all of the representative manufacturing methods that have been published to date. Currently, in a majority of the major countries of the world, G.D. Searle's utilization patents are in effect to the G.D. Searle Company and Ajinomoto, who has an agreement relationship with them, has a virtual monopoly of the market and there is very little room for an advance into the market by another company.

By the late 1960's the Ajinomoto Company had already established industrial technology and around 1975 had conducted test production. After this, for a time they ceased production temporarily, but, following FDA approval they began serious production beginning in 1982 with all of their production being exported to the G.D. Searle Company. However, since the Ministry of Health and Welfare also gave its approval in August of 1983, they have, since 1984, begun domestic marketing activities as well. After the FDA's approval, the G.D. Searle Company had been satisfying the U.S. market requirements with the Ajinomoto imports and with the output from its own plant located in the Chicago suburbs. However, in order to cope with the increase in demand resulting from the FDA's approval for its use in soft drinks, they have recently completed construction of a $100 million plant in Augusta, Georgia.

Ajinomoto and G.D. Searle have teamed up in a joint venture in Europe based in Switzerland and are conducting sales efforts there. On the other hand, in Italy, the Pierrel Company and Farmitalia Company, and in England the Angus Company have small-scale production operations of which a portion is directed to the G.D. Searle Company and the remainder is sold elsewhere. With respect to these operations, information concerning their volume and other details are unknown. In 1984, Toyo Soda completed a pilot plant for use of the fermentation method and is currently test producing and test marketing its output. They recently concluded an arrangement with the huge DSM Company of Holland in anticipation of the expiration of G.D. Searle's patent rights in 1987 and are currently planning the construction of a commercial scale plant in Europe.

As stated earlier, with respect to the various manufacturing methods, the APM production process is extremely lengthy and because the element of know-how plays a large role in the process, it is not possible to make any quick judgmental determinations from the patent itself as to which technology is the most advantageous economically. Additionally, it is necessary to factor into the equation the economic cost of the raw materials, particularly differences in cost differentials between L-body and racemized phenylalanine costs. The current market price of APM is said to be between $100-$150 per kilogram. However, because it is expected that the same degree of sweetening as with sugar for the same price or lower is potentially possible in the future, it can be assumed that there will be furious competitive competition in the future in this technology development race.

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BIOTECHNOLOGY APPLICATIONS IN PERFUMERY, FLAVOR INDUSTRIES

Tokyo BIO INDUSTRY in Japanese Nov 85 pp 5-11

[Article by Shinobu Gocho, chief researcher, Kawasaki Laboratory, Hasegawa Koryo Co., Ltd.]

[Text] The growth, transformation, etc., of aromatic substances, using microorganisms and enzymes, are already under study and part of the results have been put to practical use. Also, the use of tissue culture technology is being researched. New biotechnology is also being introduced and by using these technologies, product development is expected to become even more active in the perfumery and flavor industry.

1. Introduction

Experiments are already being conducted actively to produce substances related to perfumery and flavor through use of microorganisms, enzymes, and tissue cultures.

In this article, I intend to compile what has been reported on the growth of aromatic substances through biochemical processes and touch upon areas in which further development can be anticipated through introduction of biotechnology.

2. Growth and Transformation of Aromatic Substances Through Microorganisms and Enzymes

The growth and transformation of aromatic substances through microorganisms and enzymes are being studied from various angles. In the ensuing paragraphs, a number of them will be introduced.

(a) Growth of Aromatic Products Through Carbohydrate Sources

It is a known fact that when various types of microorganisms are grown in carbohydrate compounds, such as glucose, they emit different aromas, including fruity odors.

Lanza, et al.[1] noticed microorganisms which produce fruity aromas and selected **Ceratocystis monoliformis**, to conduct detailed studies of the armomas produced by the fungus. Changing the carbon and nitrogen sources of the growth medium, they discovered that different fruity aromas were produced through culture of the fungus and showed the possibility of producing flavor essences with fungus

For example, they discovered that by using glucose as the carbon source and urea as the nitrogen source, the fungus grown in a liquid shake culture produced a banana-like aroma. Furthermore, they have identified functionally that glucose-leucine culture produces an overripe banana aroma; glycerol-urea, canned Western pear aroma; and glactose-urea, citrus, grapefruit and lemon aromas.

Furthermore, they have conducted analyses of the aromas produced and confirmed the growth of esters, such as isoamyl acetate, n-propyl acetate, etc., r- or o-decalactone, and monoterpenes, such as geraniol, citronellol, etc. Also they have conducted research on monoterpene biosynthesis using this fungus and reported that growth takes place via the MVA [mevalonic acid] pathway as in higher plants[2].

In addition, the constituents of the peach-like aroma produced by **Sporobomyces odorus** have been reported[3,4] and the aroma produced by **Ceratocystis variospora** has been studied by many researchers.[5]

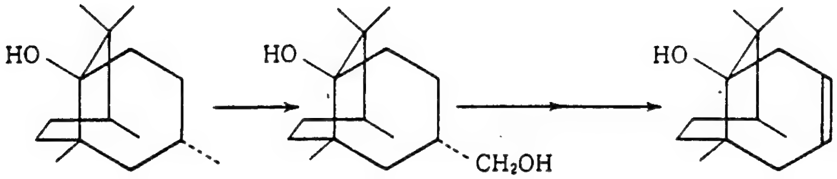
Also, Koizumi, et al,[6] have recently selected from a sap ferment, a bacterial strain which produces strong aromatic substantances and are analyzing the aromas produced by the culture media. As a result, they have identified the growth of aromatic esters, high-grade alcohol, etc. and believe that the composition ratio of these esters is one of the important factors which determine the special character of the aroma.

To convert these results into commercial products, increase of aromatic content is considered necessary and a variety of research is being conducted. In general, it is believed that monoterpene substances restrict the metabolism of microorganisms. Schindler researched the process of adding lipophilic adsorbent to the culture medium of **C. variospora** to absorb monoterpenes being produced and reported that the growth accumulate of geraniol increased approximately tenfold.[7]

(b) Production of Synthetic Perfumes

In general, enzyme reactions have high substrate and reaction specificities. Using these principles, researchers are using microorganisms and enzymes to conduct selective reactions which are difficult to perform through organic synthetic reactions and studies are being made to manufacture aromatic substances or their intermediates.

The chief ingredient of patchouli oil, which is an important perfume ingredient is patchoulol (1) but it is said that norpachoulenol (3), which it contains in minute quantity, is the main fragrant constituent (see Figure 1).



Patchoulol〔1〕 10-Hydroxy-patchoulol〔2〕 Norpachoulenol〔3〕

Figure 1. Growth Process from Patchoulol to Norpachoulenol

As a synthetic process of (3), the method shown in Figure 1 can be conceived and 10-hyroxypatchoulol (2) can be obtained through hydroxylation of (1) but this is difficult chemically. Therefore, the process was developed of converting (1) to (2) biochemically and from (2) to (3) through organic chemical reaction.[8] It has been reported that, at first, this oxidation reaction was determined to be possible with the use of rabbit and dog livers[8] but later, it was found that the process can be conducted with the use of microorganisms.[9,10]

Also, in the manufacture of intermediate bodies of musk family synthetics, which which are important perfume fixatives, the use of microorganisms has been researched.

Long-chain dicaboxylic acid and w-oxycarboxylic acid are being sought as synthetic raw materials of the macrocyclic musk, which has an excellent aroma and the structure, among the various musk synthetic perfume fixatives, most resemgling the natural musk.[11]

It is believed that n-paraffin is metabolized by n-paraffin mutant bacteria via the process shown in Fugure 2.[12]

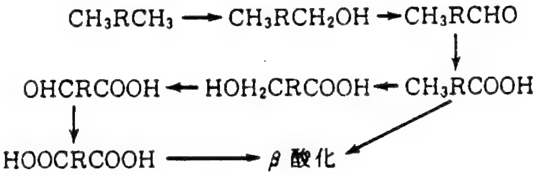


Figure 2. Oxidation Process of n-Paraffin through Microorganisms[12]

Therefore, carboxylic acid is the initial oxide of n-paraffin created by these microorganisms and by blocking enzymes which contribute to β oxidation growth accumulation can be anticipated.

Since long-chain dicarboxylic acid is relatively difficult to produce through organic synthesis, experiments have been conducted to mass produce it using microorganisms and n-paraffin as the raw material.

Uchio, et al., selected dicarboxylic acid accumulated yeast from n-paraffin mutant strain and conducted mutagenic treatment for study. As results, they have reported that they were able to grow long-chain dicarboxylic accumulates at the rate of 50 g/liter or a conversion recovery rate of 70 percent.[13,14]

A patent has been issed for incubating n-paraffin mutant bacteria by adding acrylic acid and ion exchange resin in the culture medium to increase the growth accumulates of dicarboxylic acid and w-oxycarboxylic acid.[15]

Also, recently, Uemura, et al. have reported they have succeeded in mass producing dicarboxylic acid from n-paraffin with the use of yeast and on the industrial production of brassylic acid (DC-13).[16] This brassylic acid is used as the raw material of ethylene brassylate which is one of the musk synthetic fixatives.

Furthermore, the use of microorganic enzymes in optical partitioning has been reported in one case.

1-Menthol has a refreshing taste and is used in foodstuffs, medical products, etc. This substance had been synthesized from d-citronellal via 1-isopregol but a method has been developed of synthesizing it from m-cresol via thymol. Since the substance obtained through this process is optically inactive dl-menthol, Yamaguchi, et al.,[17] have conducted further studies on optical partitioning.

Screening microorganisms which would hydrolyze dl-menthyl acetate, they selected **Rhodotorula mucilaginosa AHU 3243** and conducted studies on asymmetric hydrolytic conditions and made improvements to the bacterial strain. They have reported on the successful production of 44.4 g/liter 1-methanol with substrate density of 30 percent.

Subsequently, Omata, et al.,[18] conceived that since these dl-menthyl ester and 1-menthol were insoluble in water, the reaction process of using immobilized bacterial bodies in organic solvent would be effective and researched the conditions. In a reaction process using **Rhodotorula minuta var. Texensis IFO 1102** immobilized with urethane prepolymer, dl-menthyl succinate as raw material and water-saturated n-heptane as solvent, they reported that they obtained excellent results as shown in Figure 3.

Furthermore, they reported that the stability of the immobilized microorganic enzymes improved greatly and the half-life period of enzyme activity, which had been 2 days with free bacterial bodies, was extended to 63 days.

(c) Production of Aroma Compounds

Also being considered is the process of producing aroma compounds and bases for aroma compounds by using simple substances of synthetic aromas as the starting materials to grow various derivatives with the use of microorganisms.

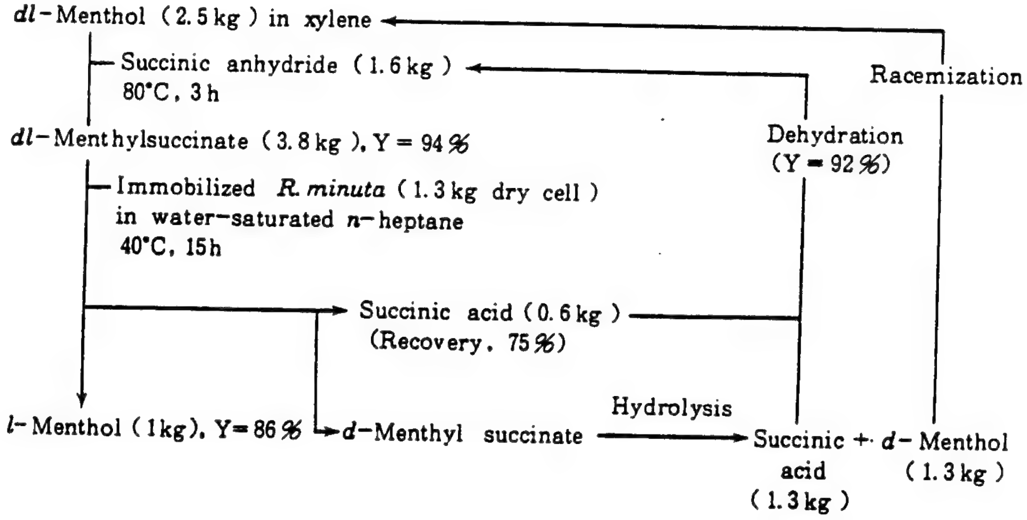

dl-Menthol (2.5 kg) in xylene
Succinic anhydride (1.6 kg)
80°C, 3h
dl-Menthylsuccinate (3.8 kg), Y = 94%
Immobilized R. minuta (1.3 kg dry cell)
in water-saturated n-heptane
40°C, 15h
Succinic acid (0.6 kg)
(Recovery, 75%)
l-Menthol (1kg), Y=86%
d-Menthyl succinate
Hydrolysis
Succinic acid (1.3 kg)
+ d-Menthol (1.3 kg)
Dehydration
(Y = 92%)
Racemization

Figure 3. Flow Sheet of 1-Methanol Production Through Immobilized Bacteria

It is believed that various compounds with a trimethylcyclohexane ring, which are considered as metabolites or degrades of carotenoids, are important in tobacco flavoring. Mikami, et al.,[19] have conducted research on microbial transformation of ionones, which are believed to be intermediates of carotenoid degradation, to obtain tobacco flavor.

After screening various types of microorganisms, they selected **Aspergillus niger JTS 191** as the useful strain and studied the transformation of ionones and resulting products. It was projected that β-ionone was transformed into the various derivatives via the pathway shown in Figure 4.

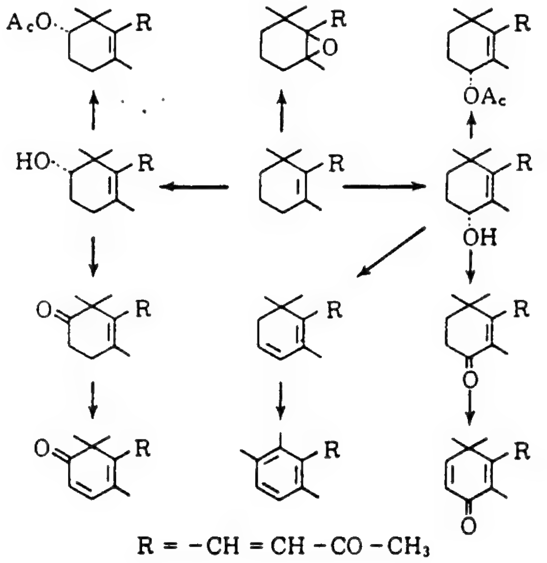

AcO
R
O
OAc
HO
OH
O
R = -CH = CH - CO - CH3

Figure 4. Projected Pathway of Transformation of β-Ionone by **A. niger JTS 191**

These converted products had a taste and flavor similar to that of tobacco and the discovery that the mixtures of the transformed substance were useful as tobacco flavoring led to commercialization.

Karube, et al.,[20] are conducting research on the immobilization of bacterial bodies with the main objective of increasing the stability of microbial enzymes in order to perform continuous conversion of ionones.

Furthermore, a patent has been applied for the process of producing aroma compounds, or aroma compound bases, with the flavor of raspberry, apricot, tobacco, etc. by following similar processes, utilizing microorganisms of **Botryodiplodia** strain, **Lasiodiplodia** strain, etc. with α-ionone, β-ionone, α-irone and β-irone as substrates.[21]

Also, research is being conducted on the microbial transformation of **cis**-Abienol,[22] Sclareol,[23] and Menthone[24] to various products using similar methods.

3. Food Flavoring Using Microorganisms and Enzymes

Not only aroma but taste is considered an integral part of flavor which is obtained commonly from animals and plants.

Much of biometabolism is performed by enzymes. When onions or garlic are ground, a peculiar odor is formed. Thus, the existence of aromatic substances in the precursor form and the mechanism they develop, whereby enzyme action takes place in the same cells through outside stimulus, are known.[25]

Also, there are many food products in which microorganisms contribute directly to the production and perform an important role in creating aroma and taste, such as brewed products, including sake, miso, soy sauce, etc. and dairy products, including cheese, yogurt, fermented butter, etc.

Based on these growth mechanisms of aroma and taste, development of various flavors, using microorganisms and enzymes, is taking place actively.

It is known that free fatty acids are an important factor in giving various dairy products their special flavors. Therefore, the use of lipase in the manufacture of dairy products was considered and the use of lipase in milk fat, etc., to produce flavor like that of a dairy product has also been researched.

It was generally considered that lipase of animal origin was good but as a new enzyme source and from an economic standpoint, microbial lipases are being researched in various ways.[26]

Kanisawa, et al.,[27] conducted research on the production of various dairy product flavors by using five types of microbial lipases on the market and reported that lipases organically produce distinctive flavors depending on their origin and, moreover, among them, the lipase produced by **Candida cylindracea** was the best.

For example, they reported that with butter as the raw material, products of a 66.6 percent hydrolysis rate using this lipase, contained approximately 53 percent free fatty acids, which is 150 to 200 times denser than the raw material butter, and by adding 0.2 to 0.5 percent of this to processed foods, butter-like flavor can be developed.

In the manufacture of cheese, microorganisms such as lactic acid bacteria, molds, etc. perform an important function and research on this flavor development has been conducted from long ago.

There are various kinds of cheese and among them, there is the blue cheese of the methyl ketone group which is noted for its strong pungent smell and taste. This distinctive flavor is developed by **Penicilliun roqueforti**. It is postulated that, in the development process of the methyl ketone group, the hydrolysis of milk fat by lipase produces free fatty acids which are then converted to methyl ketones by this fungus.

On the basis of such basic information on flavor development mechanisms, various methods are being developed using milk fat, etc., as raw materials and through a liquid fermentation method employing lipase and **P. roqueforti**, to manufacture within a short period of time, products with a high concentration of methyl ketones which resemble natural blue cheese, even organically.[28]

For example, Jotty [Jolly], et al.,[29] have reported on a liquid shake culture process, using raw cream or coconut fat as raw materials and adding lactic acid bacteria starter and lipase prepared from **Asp. oryzae** and **P. roqueforti** spores. The blue cheese concentrate thus produced was blended with skim milk retentate, obtained through ultrafiltration, and salt to produce a new blue cheese material.

In this material, the content of heptanone, which is the chief odor constituent, was 25.7 umol/g or five times greater than in natural blue cheese. Since this blue cheese material can be produced efficiently in a short period and the fat and salt contents can be freely altered, it is believed that it can be used as a flavoring material.

Also, there are cases where microorganisms are used in the manufacturing processes of ham and sausages.

Noting this point, the four companies of Ito Ham, Hasegawa Koryo, Unitika and Shibaura Engineering Works of the Fat Group of the Food Industries Bioreactor Technology Research Association, which started in 1985, are jointly engaged in a 5-year plan to develop ripened meat flavor.

In addition, various methods using microorganisms to improve flavor are being developed.

In the manufacture of concentrated raisin juice, the product obtained through the process of first, slurrying and pectinasing, then, filtering and concentrating, lacked the distinctive mellow aroma of raisin and was simple in taste Therefore, further studies were made.

As a result, it was learned that if the juice were fermented after addition of lactic acid bacteria, a fresh fruitlike aroma formed. Furthermore, when heated after injection of amino acid and monosaccharide, the distinctive raisin aroma was strengthened and a product possessing fresh fruitlike aroma was developed.[30]

4. Use of Tissue Culture in Perfumery and Flavor Industry

The production of essential oils through tissue culture of aromatic plants has been researched.

In general, secondary metabolites are difficult to form in tissue culture. Moreover, since essential oil is made up of many constituents, it is projected that it would be difficult to obtain a product idential in composition to the mother plant.

In research conducted heretofore on tissue cultures of aromatic plants, there are reports[31-33] that essential oil components were obtained but, on the other hand, there are many reports that tissue culture was possible but essential oil components could not be developed.

Although there are difficult aspects, information is being obtained that when undifferentiated culture cells were segregated to some extent, under different conditions, the accumulation of targeted substances was seen. It is believed that as the mechanisms for active manifestations of secondary metabolites become clarified, research in this field will further progress.

Studies are also being made on tissue cultures using precursors to form the desired substances or to increase the accumulates.[34-36]

With the application of tissue culture technology, progress has been noticeable in the field of plant breeding. Previous crossbreeding method was limited to closely related groups but a hybrid of distantly related varieties has become possible. Furthermore, the use of genetic alteration is being considered in this field and further advancement can be expected in the future.

Research and development of aromatic plants are also being reported.[39] It is anticipated that research would advance over a wide front, including the development of new varieties containing many essential oil components, species with improved cultivatable areas, etc.

5. Conclusion

References have been made to a number of R&D cases applying the technologies of microorganisms, enzymes and tissue cultures to substances related to perfume and flavor. Some of them have been commercialized but many are still in the developmental stage.

As far as material transformations are concerned, it is believed that with the use of the special characteristics of biochemical processes, such as stereospecific reactions, stereoselective reactions, asymmetric syntheses, etc., great progress can be expected.

Presently, the advancement and development of the technologies of genetic alteration, cell fusion, bioreactor, tissue culture, etc. are remarkable.

If these new technologies can break the "barrier," considered the limit of research conducted until now, the area in which biotechnology can be applied will probably expand in the perfume and flavor industry.

In the genetic engineering report titled, "Impacts of Applied Genetics," released by the Office of Technology Assessment of the U.S. Congress in 1981, it is predicted that substances related to perfume and flavor such as, cironellal, citronellol, geraniol, lynalyl acetate, nerol, -terpineol, -terpinyl acetate, cinnamaldehyde, etc. will enter into commercial production through the fermentation method within 20 years.

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BIOTECHNOLOGY

DESIGNING OF BIOPOLYMERS DISCUSSED

Tokyo BIO INDUSTRY in Japanese Sep 85 pp 41-49

[Article by Tatsuo Ooi, professor, Institute for Chemical Research, Kyoto University]

[Text] In order to design biopolymers, one must understand their properties and structural principles of configuration of the biopolymers. Of the representative biopolymers, nucleic acids and proteins, proteins have diverse and varied functions and structures. Based on the research results to date, I shall discuss the present state of knowledge regarding the process of biosynthesis, the formation of a three-dimensional configuration following peptide chain synthesis, and the three-dimensional structure of a protein, and what sort of designing is feasible based on that knowledge.

1. Introduction

Industrial development potentials are being sought centered on biotechnology directed toward not too distant a future. Biopolymers are the materials that play a key role in this development. Probably, it takes a specialist in the field to accurately answer what biopolymers signify. Literally interpreted, they denote in vivo polymers. However, since living organisms take in raw materials externally and produce the necessary polymers themselves, the varieties are fairly limited. In this sense, they are different from ordinary synthetic polymers. Furthermore, biopolymers produced for living processes are endowed with necessary properties accordingly, and I don't think things will work out smoothly even if we sought to use these biopolymers for other purposes. Therefore, the basis for molecular designing includes the precondition that the target molecules are those whose functions in organisms are thoroughly known. One may think that although he does not thoroughly understand the theory, he has knowledge of enzymes that catalyze chemical reactions and he can probably design such proteins. However, careful thought would soon make him realize that such designing is not so easy. It is wise, after all, to reevaluate the work on the basis of reasonable knowledge regarding biopolymers.

A living organism matures through a process of differentiation and growth, and it constantly repeats metabolic processes. This means that the organism does not incorporate the polymer from the external environment in that form, but has it ready in a low molecular weight form and connects these monomers as needed to use as so-called biopolymers. At the same time, when they become unnecessary, they are degraded to their original materials or when the resultant degraded substances are not beneficial to the organism, they are excreted. In other words, ordinary biopolymers cannot be made very stable. Of course, there are some that seem exceptionally stable. The seeds of plants that are more than 1000 years old have been preserved, or bacterial spores are strongly conditioned against environmental changes and are also less susceptible to radiation damage. The characteristics acquired by living organisms over several billion years are broad and deep. Consequently, unless we expect stability to function even under extremely severe conditions, I believe there is a fairly extensive range of biopolymers already in existence that are able to meet our purposes. In the lifestyle prior to the time humans devised the system of industry to process materials, biopolymers were the support of the lifestyle. Silk which is a protein, cotton that comes from plant seeds, wood, food, etc. are all familiar to us.

However, in order for us to be able to design these biopolymers, we must understand the production process in vivo and the principles of molecular configuration. We have the knowledge regarding the raw material such as water, carbon dioxide, ammonia, and the final products obtainable by providing energy necessary for processing; and we also know the actual results as observed in agriculture and forestry. However, regarding the intervening processes, there are still many parts in the dark. Under such conditions, there is no way that designing is possible. Then, regardless of the goal, the problem is restricted to proteins whose principles have been revealed albeit imperfectly. When speaking of designing a biopolymer, the present stage is nowhere near the designing of fibers, wool, or wood. If one expects new things by these designs, trial and error is the only way.

2. Biosynthesis of proteins

Proteins, one of the biopolymers believed to be promising for designing, have in fact marvelous actions, i.e., functions. Both the ability to manufacture biopolymers and the ability to break them down which are inherent in living organisms are in fact due to proteins. In addition, the fact that proteins are always used in key places in both motion mechanisms and various sensory mechanisms truly demonstrates the diversity and effectiveness of biopolymers. The research in molecular biology that has been advanced in the last 20-30 years, revealed how proteins are produced from the information source of proteins, DNA.

In the field of biochemistry, studies have been concerned as to how proteins are localized and how various functions relate to structure. And, it may be said that the routes are roughly elucidated. As a consequence, there are prospects for the manufacturing of growth hormone and interferons in large quantities. However, what we must caution against is overconfidence, and it is necessary to keep in mind that there are many parts of the black box that are as yet poorly understood.

Although synthesis proceeds as if no doubt exist when a textbook-like description of the process is made, in fact there are some problems at every stage. However, I shall discuss the synthesis pathway here without pointing out these problems assuming that things proceed smoothly. Fig. 1 shows the schematic diagram of protein biosynthesis[1]. The base sequence of a DNA (deoxyribonucleic acid) polymer composed of four kinds of bases, A, T, G and C, is copied into RNA with an enzyme called RNA polymerase. The information of the sequence is transfered intact to RNA. Of the RNAs, tRNA assumes the well-known secondary structure of a clover leaf as well as an L-shaped three-dimensional structure due to its specific base sequence. Then, with the help of aminoacyl synthetase, it becomes aminoacyl tRNA with an amino acid attached to its 3' end. On the other hand, rRNA (ribosomal RNA) along with ribosomal proteins form large and small ribosomes, creating the site for biosynthesis. The amino acid sequence for proteins is written in the base sequence of mRNA, and three bases as a set correspond to one amino acid. In other words, 20 amino acids and stop signals correspond to 4 x 4 x 4 = 64 different arrangements. The table of codons shows how they correspond, and all living organisms on earth use a common codon table. Although it may seem insignificant, it is a very important fact. The reason why it is possible to have E. coli manufacture human growth hormone is that E coli carries out biosynthesis with the same codon table as man. It is interesting to see whether the spacemen in science fiction use the same codon table as earth creatures. With the codon table in hand, translation begins from the formyl methionine codon, AUG, with every three bases on the mRNA coding for an amino acid, and with the stop codon, synthesis of a polypeptide is completed. The DNA information is expressed as a polypeptide chain of protein in this manner.

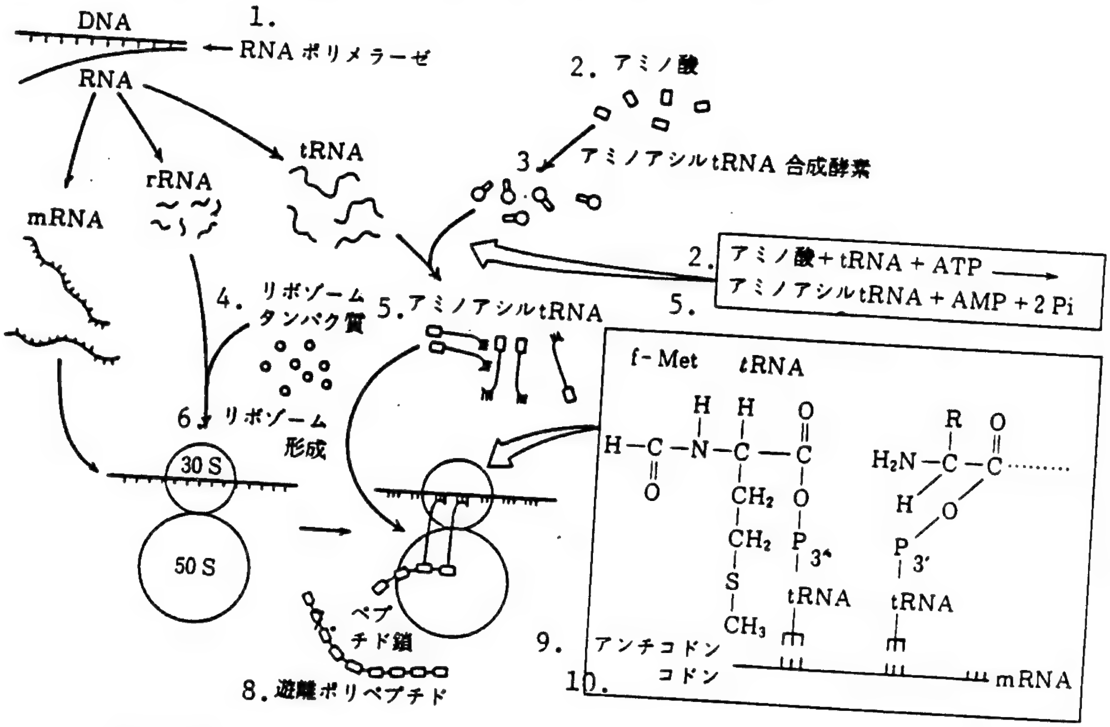
DNA
1.
RNAポリメラーゼ
RNA
2. アミノ酸
3 アミノアシルtRNA合成酵素
tRNA
rRNA
mRNA
2. アミノ酸＋tRNA＋ATP → アミノアシルtRNA＋AMP＋2 Pi
5.
4. リボソームタンパク質
5.アミノアシルtRNA
6 リボソーム形成
30 S
50 S
ペプチド鎖
8.遊離ポリペプチド
f-Met tRNA
H—C—N—C—C
CH₂
CH₂
S
CH₃
P
3'
tRNA
H₂N—C—C
R
H
O
9. アンチコドン
10. コドン
mRNA

Fig. 1 Schematic diagram of the process of protein biosynthesis

key:			
1)	RNA polymerase	2)	amino acid
3)	aminoacyl tRNA synthetase	4)	ribosome protein
5)	aminoacyl tRNA	6)	ribosome formation
7)	peptide chain	8)	free peptide
9)	anticodon	10)	codon

The process of biosynthesis described above may be considered virtually accurate and even in exceptional cases, only a certain amount of ambiguity exists. However, there are many things that are not understood in detail.

For example, even though all the substances shown in Fig. 1 are at hand, one cannot freely make proteins in vitro. This is the reason for borrowing the biosynthetic mechanism of bacteria such as E. coli and having them transcribe and translate the DNA information externally for the protein sought. No doubt, in the near future, a large-scale biosynthesis mechanism will be established.

In order to design biopolymers and produce their molecules, at the present time, one must rely either on chemical synthesis or having organisms manufacture them by using the above-mentioned biosynthesis system. However, aside from mass production, if one can in fact obtain designed molecules, the value of the designing is justified because one can then prove that designing is not sheer nonsense. Consequently, unless designing is carried out by taking the construction process into account, the molecules designed will not result. In order to accomplish this, several conditions must be met. If a biosynthesis system is used, it must pass the condition that the media environment is water. In Fig. 1, to make RNA, the molecular units of ribonucleic acid must move in a solution, and when amino acids attach to tRNA, they must be in a solution. This is only a matter of course based on the fact that proteins themselves are present in a saline solution of about 0.2M. However, if one plans to design electronics-related biopolymers where electrical conductivity is the issue, one cannot ignore this fact. If one wish to avoid water, he may use a chemical synthesis in an organic solvent system, for example, a well-known solid phase method known as the Merrifield's method. However, the reliability of attaching amino acids is far inferior to the biosynthesis system, and even when synthesis is achieved as intended by the sequence provided, it is difficult to obtain a chain with the correct sequence. It is especially difficult to obtain a long chain with more than 100 residues. Consequently, the desired substance must be separated and purified from by-products.

3. Properties and characteristics of biopolymers

As has been stated, one cannot design molecules without the knowledge of molecular structure and mechanism. Even if one is convinced that it is possible in principle to construct a polypeptide chain having a certain designed amino acid sequence, with what properties that molecule is endowed depends on its behavior following the biosynthesis of the polypeptide chain. A polypeptide chain synthesized under the fairly restricted conditions of an aqueous environment which is a problem, the presence of low molecular salt ions, and the temperature and pressure in which organisms survive results in a certain conformation. Moreover, this conformation in the natural state is the basis of the biological functions expressed. Although our knowledge of chain folding is not adequate at present, it is known from denaturation and renaturation experiments in vitro that no mystic power is at work, but it is in the category that is explainable using common sense thermodynamics or statistics. However, this does not mean that we understand the chain folding, and the elucidation of this process is still being studied as an important subject.

By stating under limited conditions, what I wished to point out is the fact that organisms live under very extreme conditions. For example, it has been confirmed that organisms exist even on the ocean floor of 5,000 meters or more, and it is likely that they exist in even deeper seas. This means that the above-mentioned biosynthesis is possible even under a high pressure of about 500 atmospheres. It is also a well-known fact that there are thermophilic bacteria that proliferate in hot springs near the boiling point at 90°C or more. Furthermore, there is even a report confirming the presence of organisms in the environment of high pressure and high temperature near a submarine volcano. Consequently, if the component molecules are properly selected, the mechanisms of biosynthesis and folding can tolerate fairly severe conditions. However, that substance is unknown.

Now, assuming that the three-dimensional structure of protein molecules were formed, what is the characteristic of that property? It is that proteins take a given natural conformation and function as determined by the conditions in which the organisms inhabit. Enzymes are frequently quoted, easy-to-understand examples: they catalyze specific chemical reactions. The special features here are the fact that they are "specific" and highly efficient catalyts. In other words, the specificity of identifying substances involved in chemical reactions is a special property of biopolymers, especially proteins, and it is not restricted to enzymes, but the recognition ability found in antibody-antigen reactions also depends on the same mechanism. In addition, whereas the catalytic efficiency of small molecules is considered outstanding at 10-100 fold, the normal values for enzymes is over 10^5. One of the feasibilities focused on biotechnology is probably the design and production of substances with such superb recognition ability and high efficiency. The fermentation industry such as breweries has indeed utilized these special properties.

As far as enzymes are concerned, the three-dimensional molecular structure is known for a considerable number of them, and except for details, studies are under way to explain their mechanisms at the molecular level. Although our level of understanding is insufficient, the specificity inferred from the three-dimensional structures of enzymes and the essence of the catalytic mechanisms are as follows: first, at the binding stage of the substrate molecules added to a chemical reaction, there must be a binding site on the surface of the enzyme molecule. The formation of the central site, the active site, is closely related to folding of the polypeptide chains, which is a point in common for all enzymatic proteins whose stereostructures have been elucidated. The technique applied separates the chain into two parts to form a dent on the molecular surface where the substrate molecule can bind, and after each part forms a mass as a domain, those two masses are catalyzed. One may imagine a similar situation in which two balls in contact create a dented area. In the case of enzymes, the special feature is that this dent serves as a mold for the substrate molecule. In other words, the basis for proteins recognizing a specific molecule is the conformation matching as in a key and a keyhole. In the case of enzymes, the site of a chemical reaction must be created in addition to the matching characteristic. This is accomplished by placing, at the site of the key reaction, a polar group to which electrons are readily transferred. This is a spatially delicate structure in which the wonder of the most important and seemingly mystic three-dimensional structure of protein is found.

The above example is merely one face of proteins. We cannot generalize the diverse functions and varied structures of proteins based on enzyme research. However, we learned of an example of what the resultant state is when natural conformation occurs based on some sort of principle following the production of a single polypeptide chain. Moreover, the amino acid sequence of the polypeptide chain is indeed what determines the final conformation. This is shown by the fact that diverse enzymes are produced by changes in the amino acid sequence. Then, would it be possible to design enzymes?

4. Designing enzymes

Once we understand the fact that the information for a protein is coded in DNA, the biosynthesized chains spontaneously take their natural stereostructure and, if it is an enzyme, it demonstrates enzymatic activity, the natural sequence is to attempt to synthesize polypeptides having any amino acid sequence to create new enzymes. Many organic chemists first attempted the total synthesis of proteins. In the latter 1960, when the synthesis of insulin and ribonuclease A began while verification purity was repeatedly performed with the usual liquid phase method, Merrifield in the United States was developing a solid phase method. Synthesis of relatively short peptides of several tens of residues was not that much of a problem using the liquid phase technique. However, in the case of a long chain, solubilization of synthesized fragments impeded the synthesis process slowing the research progress. Thus, the solid phase method demonstrated its power, and its basic principle has been passed on to the present DNA synthesizer. Using this method, Merrifield succeeded in total synthesis of ribonuclease and ribonuclease A with 124 residues. As has been pointed out, reliability of over 90 percent cannot be expected with a normal chemical reaction system, and the components corresponding to ribonuclease A had to be separated and purified from the polypeptide chain formed. It was also difficult to ascertain whether the purified substance truly had the correct sequence. However, the substance finally obtained had enzymatic activity and no difference was found from the natural protein. Whether the synthesis was from C-terminal or it required purification, we found the prospect for designing and producing enzymes.

The report2) on a synthetic polypeptide, which is a simplified ribonuclease S-protein, published 10 years ago in 1975 was perhaps one of the earliest among enzymatic designs. The strategy then used by the designer, Gutte, is not too different from what is being conceived today. Look at Fig. 2. This is a three-dimensional diagram of the stereostructure obtained from a crystal analysis of the above-mentioned ribonuclease. Although the actual molecule has a structure densely packed with component atoms, the structure is shown with lines connecting alpha-carbons in order to show the folding outline of the main chain. If you look at the right side diagram with the right eye and the left side diagram with the left eye, a three-dimensional image should appear. The active site, a key factor for enzymes, is the pocket formed with His12, Lys41, and His119. Consequently, Gutte's idea was that the outer portion far from the active site was unnecessary, and one way is to make a polypeptide chain produced by connecting the bold lines in the diagram. Fortunately, there are many studies on ribonuclease A: this protein is cut by a digestive enzyme called subtilisin at the peptide bond between the 20th and 21st residues and separated into a 1-20 S-peptide and a 21-124 S-protein. Interestingly, these two parts are known to be conjugated in a normal environment and have exactly the same activity as in the natural state. Gutte deleted 104 residues of S-protein and designed the 70-residue peptide shown in Fig. 3. If this S-protein replacement were to have a structure similar to the natural stereostructure, unless proper residues are added in the deletion sites, the chain is unable to form a short-cut. Thus, several excess residues are inserted.

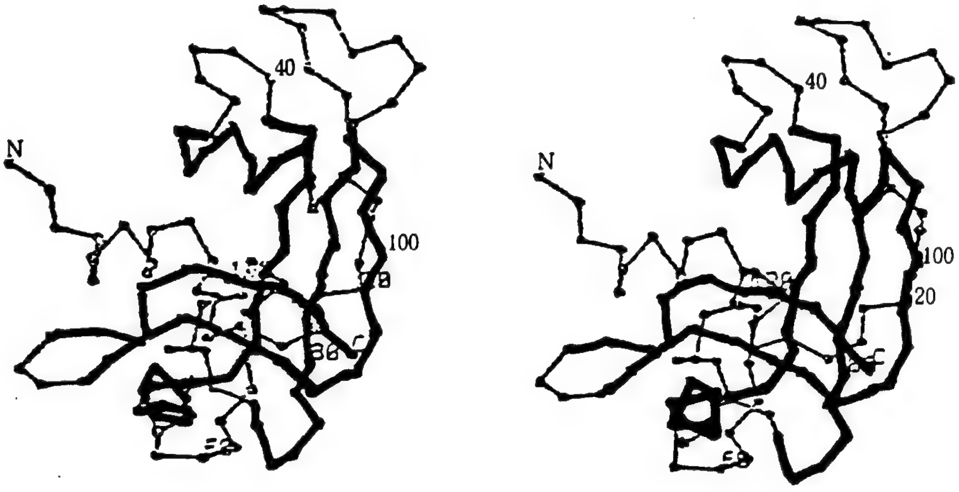

Fig. 2 Three-dimensional diagram showing stereostructure of ribonuclease A (Bold lines correspond to 70-residue protein)

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S-protein :        21 S S S N Y C N Q M M K S R N L T K D R C K P V N T   45
                                L N Q M M K S R N L ——— A — K P V N T

S-protein : 46    F V H E S L A D V Q A V C S Q K N V A C K N G Q T N C Y Q S   75
                  F V H E S L A D V Q A V —————————————— A A ——————————— Q S

S-protein : 76    Y S T M S I T D C R E T G S S K Y P N C A Y K T T Q A N K H   105
                  Y S T M S I T D A R E ———— G —————————— Y K T T Q A N K H

S-protein :106    I I V A C E G N P Y V P V H F D A S V   124
                  I I V A A E G ——— Y V P V H F D A S V
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Fig. 3 Comparison of the designed amino acid sequence of the 70-residue protein and the sequence of S-protein

According to the report, this synthesis of a designed molecule was successful, and when S-peptide was added, enzymatic activity was demonstrated. This is one of the examples of laboratory scale designing carried out after it became possible to make a long peptide chain by chemical synthesis. However, the manufacturing of proteins is difficult by chemical synthesis method. The reason is that the amino acid sequence of the finished molecules is not very reliable. However, progress has been made recently in a technique for producing proteins using a biosynthesis system. In this case, designing an amino acid sequence leads to a DNA design, and the reliability of the sequence of the product is as high as with live organisms, necessitating no special concern. The designing principle currently used is still based on the known stereostructure. One example may be the report[3)] of changing the substrate specificity of trypsin. After testing the bond state of a substrate molecule, the side chain is changed. In other words, amino acids are substituted to examine their effects on changes in specificity. The widespread experiments of the so-called amino acid substitution at designated sites are a consequence of recent progress in biotechnology.

5. Primary and higher-order structures

The fact that the proteins of a certain amino acid sequence results in a stereostructure dependent on that sequence has been confirmed in vitro. Then, one should be able to correctly predict protein structures and functions from such information, i.e., amino acid sequence. Furthermore, there must be some structural principle. Nature carries out this task so easily. However, despite repeated efforts by many researchers, no method has been established to correctly predict the stereostructure of a protein from the knowledge of its primary structure alone. This means that we have not yet fully understood the principle of formation of protein stereostructure. Naturally, there may be a case of accidentally deriving a protein structure in the course of various trials, but this is not a solution to the problem. If this method of deriving structures allows us to deduce structures of other proteins in a similar manner, the future is not that dark. Proteins are fairly individualistic, and what works with one protein may not be at all useful for another.

Let us look at this problem from a little more fundamental standpoint. I have already stated that no mystic power or substance is required in the process of spontaneous formation of a stereostructure from biosynthesized chain. This process is a problem of statistical thermodynamics. Then, if the intramolecular energy of a protein molecule is calculated, perhaps the natural structure may be obtained as the minimum energy state. The calculation of such energy is actually possible in the modern era in which a high-speed large capacity computer can be used. This is done by adding up all interactions among the atoms composing the polypeptide chain and obtaining them as the intramolecular energy dependent on conformation. However, it is obvious that the natural conformation state cannot be obtained directly from this. The reason, as I repeatedly pointed out, is that the environment in which biopolymers are placed is not taken into account in the above-mentioned energy calculations. It is an unshakable fact that factors such as the aqueous environment containing low molecular ions, temperature, and pressure have great influence on the conformation of the polypeptide chain, and only by taking these into account, does the prediction of a natural stereostructure becomes possible in principle. They have very complex effects but are unavoidable barriers as long as the prediction of conformation is concerned. However, this does not mean that there is no bright prospect to overcome this barrier.

If the prediction of stereostructure using energy calculations is not reliable at present, the only remaining possibility for the time being is a method using proteins with known stereostructures as the starting point. Trying to look for alternative possibilities by displaying molecules using graphics is a natural point of return. I would point out that the effect of volume elimination based on the area of repulsive forces in atoms is useful in attempts of energy calculation. This may be understood from the fact that conformation matching is the basis of specificity in enzyme molecules. Under such circumstances, the method that comes to mind is to collect as much protein data as possible for analysis and not by orthodox theory but empirically draw inferences. In other words, setting the structural principle aside, the idea is to study what sort of conformation results from a peptide chain with a certain amino acid sequence. As a precondition of the structural prediction, we assume that if the amino acid sequences are the same, the resulting conformations are the same. If this is not so, prediction is not possible in principle. However, it is not yet clear as to the applicable range of this premise with respect to the length of the peptide chains. It is known that it holds in proteins with 100 residues, but does not hold in peptide chains with 5 residues. Probably, more than 10-20 residues are necessary. Or, it may be longer.

Recently, homology among proteins has become the subject of study, and it has been elucidated that proteins having not totally identical, but similar [amino acid] sequences form very similar stereostructures, which has become a tool for studying the stereostructural relationship between primary structure and stereostructure. If homologous proteins are considered not only from the angle of molecular evolution, but as nature's experimental results of amino acid substitution, they are fitting targets for structural studies. As data accumulate in this manner, a clue may be obtained from information analyses for the structural principles, and on the other hand, empirical rules may be derived. This has become one research trend.

6. Biopolymers and their molecular aggregation

Discussions thus far have been concerned with protein molecules that exist singly and did not touch on the molecular aggregation that plays an important role in living organisms. However, the properties of biopolymers on which we must focus our attention in the future are the actions of the complex systems composed of proteins and other glycolipid molecules, lipids and nucleic acids. For example, the sensory organs are various kinds of very sensitive detectors. Their abilities to detect light, sound, chemical substances, etc. are extraordinary. The first step in detection is their interaction with the external world: in the case of light, rhodopsin, in the case of sound, hair [cell] molecules, and in the case of chemical substances, their receptor molecules receive information and convert it into electrical signals through some conversion mechanism. It is noteworthy that despite the fact that it is a site in which water molecules are involved and conductive ions are present, electrical signals are the means of information transmission in vivo. The parts in which biopolymers and electronics are directly related are the molecular aggregations such as these, which are fairly high level functions of the body. Even in the case of a highly ordered complex system, for example, the ribosomes shown in Fig. 1, where electronics is not directly involved, more than the actions of individual molecules are demonstrated. What we must pay attention to is the mechanism manifested from these higher order structures and the role of the structural molecules.

Based on recent studies, it has become possible to explain that some proteins are involved in regulation, which is the inhibitory or activating mechanism of ligand molecules on enzymatic functions. The oxygen adsorption phenomenon of hemoglobin is a good example. Or, the regulatory mechanism of muscle contractions by calcium ions results from a troponin-tropomyosin system, and the binding of calcium ions to troponin triggers the resulting mechanism of muscular contraction. In other words, the interaction of the proteins produces new functions of regulation or control. The typical function of living organisms is found in such complex molecular systems rather than enzymes where the action of a single molecule is conspicuous. Consequently, although the molecular designs in Figs. 2 and 3 are justifiable from the viewpoint of enzymatic actions, they are not necessarily on target from the viewpoint of control of enzymatic actions. On the contrary, since the part considered to be unnecessary was deleted, there is the possibility that an interaction with others that may have been present to begin with was eliminated. As can be seen from this example, the substance of design varies depending on what the goal is.

Speaking of complex molecular systems, the living organisms have to make them on their own. And, proteins in accordance with the information coded in DNA are the only source; the proteins produced must have the ability to form complex systems on their own. As can be understood from these theories, the formation of protein stereostructure is indeed an ingenious mechanism, and the command for manufacturing the necessary proteins in the required amount is properly issued at the development and differentiation staged. It has been found that this command is at times a protein. For instance, hormones are a typical example. Protein monomers produced are polymerized to become a polymer. Muscle fibers, flagella, microtubules, or the capsules of a phage, etc. all result from polymerization. In the designing of biopolymers, it is probably necessary to take even these stages into consideration. However, as stated before, the method of trial and error must be used at this stage.

7. Designing a molecule

To what extent the captioned problem can be solved depends on how deeply we understand living organisms. Furthermore, when you speak of designing, there must be a purpose: one cannot design with a vague idea. Due to numerous biochemical studies available, if one wishes to use a chemical reaction system in vivo, the design barriers will be relatively low. As the stereostructure data on various kinds of enzymes continue to increase each year, it is advantageous that the selection of reference molecules is easier. However, if one wishes to use a more complex system as the target and design the formation of a complex, very careful investigation is necessary because of the high percentage of unknowns. This does not mean that attempts in this direction are meaningless. Rather functional diversity is much broader in this case and it is probably easier to meet the designing purpose. It is an area in which future development is anticipated.

The terminology of protein engineering4) is gradually settling in. It may be defined as biopolymer designing and production. I believe there are many who may be interested in whether its application would reach solid electronics. Examples are found in feasibility studies of biocomputers and biochips. In view of the fact that biopolymers require an aqueous environment, this possibility is fairly low. This is a contrasting view to the bright outlook of electronics involving solution systems. However, in examining the properties of biopolymers produced and the state of further reduced water content, it seems unnecessary to give up from the start. Properties and biological functions related to electronics are frequently found such as the highly bipolar alpha-helix, bones demonstrating a large piezo-electric effect, or magnetized cells which are the cause of the homing instinct in pigeons, etc. Probably there are functions still unknown to us. Consequently, I believe that the possibilities grow depending on the target of what is being sought. At any rate, the important subject controling future development is more knowledge of living organisms.

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ENERGY

DIRECTIONS OF TECHNOLOGICAL DEVELOPMENT BY ELECTRIC INDUSTRY

Tokyo ENERUGI FORAMU in Japanese Jul 85 pp 84-87

[Article by Keiji Narimatsu, chairman of the Technological Development Committee, Central Electric Power Council: "Long-Term Technological Development Strategies of the Electric Power Industry"]

[Text] The mission of the electric power industry is to provide a stable supply of reasonably priced, high quality electricity, while paying attention to public safety and the preservation of the environment.

It is not going too far to say that the electric power industry found no alternative but to depend on technological development in order to achieve this mission. This was because of the two oil crises in addition to limited energy resources and tightened pollution control by the public.

Looking back to the past, at the time of the second oil crisis triggered by the Iranian revolution in 1979, everybody thought that the age of $100 per barrel of oil was imminent. Therefore, every country in the world made efforts to step up the development and introduction of substitute energies for oil and energy saving. Additionally, the world oil demand reduced greatly due to the world-wide economic stagnation, resulting in changes in the energy supply and demand structure.

With reduced oil demand oil prices have dropped recently to $27-28 per barrel from the peak of $34 in 1980, since even the Islamic God appears to have failed to stand against the economic rules. However, viewing from the unstable Middle East situation and the expected recovery of the world economy from an intermediate- and long-term viewpoint, there is concern over the possibility of stringent oil supply and demand conditions and rising energy costs occurring again in 1990's.

Under these circumstances, the electric power industry in Japan, a [resources] poor country, has stepped up the development of alternative energies for oil, including the utilization of atomic power technology and energy saving through the cooperation between the state and the people. Moreover, while the prospect of technology to utilize natural energies such as sunlight and wind power has increased in probability, the energy situation and economic conditions mentioned above have greatly changed.

In these changing circumstances, strategies have been formulated to meet social demands to ensure the security of energy, to cope with the growing costs and to clarify the direction anew--i.e., how to choose development projects on a priority basis and how to push them forward, and which technological development should be implemented with the aim of building up the vital physical prospects of new technological enterprises.

1. Basic Concept

Since Japan is poor in energy resources, it is necessary to secure technology aimed at harmonizing security with cost. In this connection, the electric power industry has to tackle:

1-1 Technological development to ensure the stable supply of energy and economical efficiency.

1-2 Technological development to hold down cost increases.

1-3 Technological development to cope with changes in social conditions.

In carrying out these development projects the electric power industry should define technological development strategies concretely by making proper evaluation and selection, promote these projects independently more than before, and aim at upgrading its own technology, but through coordination with all parties concerned.

2. Direction of Technological Development

The direction of technological development by the electric power industry is shown in the chart on the following page, but its details are as follows.

2-1 Technological Development To Ensure a Stable Supply of Energy and Economical Efficiency

In terms of oil, the ratio of the world's deposits of energy resources are estimated to be: natural gas 0.8, coal 6.2, and uranium 1 (50 due to the utilization of fast-breeder reactors) as against 1 for oil. Although the prices of oil and LNG seem to be stable at present, half of the deposits of both will be used up in 20-30 years. Thus at the present pace of consumption their prices are predicted to inevitably soar.

Taking into account economic efficiency, stability of fuel supply and operation, and from the standpoint of the best mixture, the component ratio of electric power resources in the year 2000 is predicted to be: atomic power 30-40 percent and coal 10-20 percent. It is, therefore, necessary to establish atomic fuel recycling technology, including plutonium utilization, and the utilization technology of fast breeder reactors and coal.

2-1-1 Utilization of Atomic Power Technology

With respect to light water reactor-related technology, the development of technology to expand capacity, adjust output to power demand fluctuations, and to use high burn-up fuel will be stepped up centering on the Japanese-type high performance light water reactors. These are aimed at achieveing higher reliability, safety, and economical efficiency.

With respect to the atomic fuel recycling technology, technological development will be carried out concerning uranium enrichment, the reprocessing of spent fuel and the treatment and disposal of radioactive wastes. Also, related nuclear reactor disposing technology will also be developed. Furthermore, the development of a fast breeder reactor will be stepped up to effectively utilize uranium resources.

2-1-2 Coal Utilization Technology

As the central elements for the coal utilization technology in the future, the following developmental programs will be implemented: Coal gasification composite power generation is regarded to be highly efficient and superior in environmental fitness; CWM (slurry) technology is designed to enhance the ease of handling; high performance (ultra supercritical pressure--USC) thermal power technology aimed at increasing generating efficiency; and the technology to effectively utilize coal ashes is expected to be generated in bulk in the future.

The future technological development of atomic power and the utilization of coal calls for substantial technical abilities, funds, time, and risks during the period from basic studies to practical use. This is true both through the developmental and experimental stages, and these should be conducted effectively by defining development systems and the share of responsibility right now.

2-2 Technological Development To Hold Down Costs

2-2-1 Reduction in Nuclear Power Generation Cost

A light water reactor improvement and standardization program will be implemented to reduce capital costs by carrying out the standardization of design and execution, the adoption of new construction methods and the cutting down of the term of work. Further, continuous operating time will be extended by securing higher fuel burnup, longer useful equipment life and the technology to diagnose its deterioration. At the same time, efforts will be made to improve facility utilization factors and increase generated energy during the useful life of a plant by reducing the time spent on regular inspection.

2-2-2 Load Leveling Measures

In order to increase midnight power demand and to contribute to holding down energy generation through new electric power development, regenerator-type cooling and heating systems and heat reserve systems for hot water supply will be promoted. From a long-term standpoint the development of various energy accumulation technologies, including new type cells for storing electric power, will be important.

2-2-3 High Efficiency Power Generation System

The technology concerning fuel batteries and a combined cycle power generation system will be developed. The latter is expected to be more efficient than the existing power generation systems and generally, less costly.

2-2-4 Reduction in Construction Costs

Capital costs will be reduced through the standardization of designs of generating facilities as a whole and their execution, the adoption of new construction methods, and the shortening of construction time.

2-2-5 Full Utilization of Existing Facilities

With respect to the existing light water reactors, their useful lives will be extended through the development of deterioration-diagnosing technology and a system utilizing operations and maintenance data.

Moreover, equipment diagnosing technology aimed at extending the life of equipment will be developed to center on oil thermal power generating plants which contain equipment already exceeding life expectancy. Developments will also be carried out concerning the technology to diagnose equipment so as to cope with the spread of new insulating methods, including gas insulating equipment in flow systems.

2-3 Technological Development To Cope With Changes in Social Conditions

2-3-1 Dealing With Information Society

In order to properly cope with the new direction of a new information society and to improve the quality of information, basic technologies such as advanced information transmission systems including image transmission and data banks will be developed. Also, technologies to control apparatuses and machines in the distribution facilities and to manage data flows will be developed.

2-3-2 Demand Technology

More effective and economical utilization of technology will be developed taking advantage of the safe, clean, and convenient characteristics of electricity, and in accordance with the real state of affairs in households, offices, and industries.

2-3-3 Technology for Introducing Scattered Power Sources

Technological conditions necessary for the systems to link the existing systems with cogeneration (dual purpose electricity and steam generation) plants, fuel cells, and solar light electric conversion plants and protection for such systems will be identified as early as possible.

2-3-4 Environment Preservation Technology

Technology with respect to exhaust gas treatment, thermal effluent disposition, and coastal hydraulics will be established.

Developing the technology to utilize natural energy such as geothermal, sunlight, and wind power as local and complemental, while keeping an eye on the future costs and progress in such technological developments.

3. Development System

With respect to a big project extending over a long term, development will be carried out under the guideline, "the state will lead basic research. However, the research, which arrives at experimental stages, will be continued under the leadership of the electric industry through the proper cooperation between the government and the people, taking account of its necessity and character."

Therefore, the basic research will be financed mainly from national funds, but in necessary developmental research projects, the electric power industry will also participate positively and independently with the aim of upgrading its own technology.

In order to set up such a developmental system efficiently and on a priority basis, the "technological development coordination committee" consisting of managers of companies concerned has been established and this in turn will set up a system to make general coordination between sectors of the electric power industry and to map out guidelines and plans for technological development in the industry.

Taking the above into consideration, opinions, requests and proposals will be submitted to the government (Agencies of Natural Resources and Energy, Industrial Science and Technology, and Science and Technology) and the organizations concerned (the New Energy Development Organization). Also there will be a positive effort to listen to the opinions of people and to learn from these experiences and through consultations with them.

4. How Technological Development Will Progress

Development strategies will be formulated. These will place emphasis on the preparation of scenarios for important tasks and keep in touch with the government and organizations concerned from a long-term standpoint and in keeping with the directions of the above mentioned technological developments. At the same time, technological development strategies will be reviewed on a timely basis, taking into account changes in situations surrounding the electric power industry, the progress in technological development, and cost fluctuations.

Although the contents of the long-term technological development strategies are as mentioned above, one additional remark with respect to technological development follows.

Directions of the Main Themes of Technological Development by the Electric Power Industry

				(Commercialization Development Target time)		
				1990s	Around 2,000	After 2,010 on
Technological development for ensuring stable energy supply*	Atomic Power	Light water reactor improvement, standardizing	A-BWR A-PWR	◎		
		Nuclear fuel cycle technology	Uranium enrichment, reprocessing, wastes treatment and disposal	◎		
		Nuclear reactor disposing technology			◎	
		Fast Breeder reactor				◎
	Coal	Coal gasification composite power generation			◎	
		CWM(slurry), USO (ultra supercritical pressure), utilization of ashes		○		
	Reduction of atomic power generation cost	Reduction of construction cost	Standardization of designs and execution, adoption of new construction methods, etc.	◎		
		Utilization factor improvement	Rationalization of regular inspection, extension of continuous operation time	◎		
		Reduction of fuel cost	Development of high burnup fuels	◎		
	Load levelling measure	New cells for power storage			◎	
		Midnight demand technology	Regenerater type cooling and heating systems, etc.	○		
Technological development for holding down cost increases	High efficiency generation system	Fuel cells		◎ Phosphoric acid type	○ Molten coal oxychloride	
		High efficiency gas turbine		○		
	Construction cost reduction		Standardization of designs of electric power equipment and execution, adoption of new construction methods, etc.	◎		
	Full utilization of existing facilities		Diagnosing technology of equipment such as atomic power and thermal power equipment, generators, transformers, etc.	◎		
Technological development for dealing with changes in social conditions	Response to information society		Information systems, data banks, etc.	○		
	Demand technology		High temperature heat pumps for industrial use, full electrification, home automation, etc.	○		
	Technology for introducing scattered-type electric power sources		Linking methods with systems, protection systems, etc.	○		
	Environmental measures		Exhaust gas treatment, thermal effluent measures, etc.	○		

*World energy resource deposit ratios(in terms of oil)

Oil	1
Natural gas	0.8
Coal	6.2
uranium	1

(30 due to utilization of fast breeder reactors)

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Note: ○ Priority
◎ Top Priority

I would like to stress the fact that technological development is indispensable for the industry to vitalize enterprises, just as scientific technology had a great impact on the society in the past, leading to its development and vitalization.

For instance, if reduced construction costs and load leveling technology could favorably affect the management of an enterprise through the improvement of the quality of technology, it would no doubt provide the industry with "bread," namely, cost saving. Furthermore, the technological development will provide company employees realistic "bread," but also "dreams," a big loaf of bread, to dream--they will be constantly pursuing new things for the future in the course of performing their routine duties.

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